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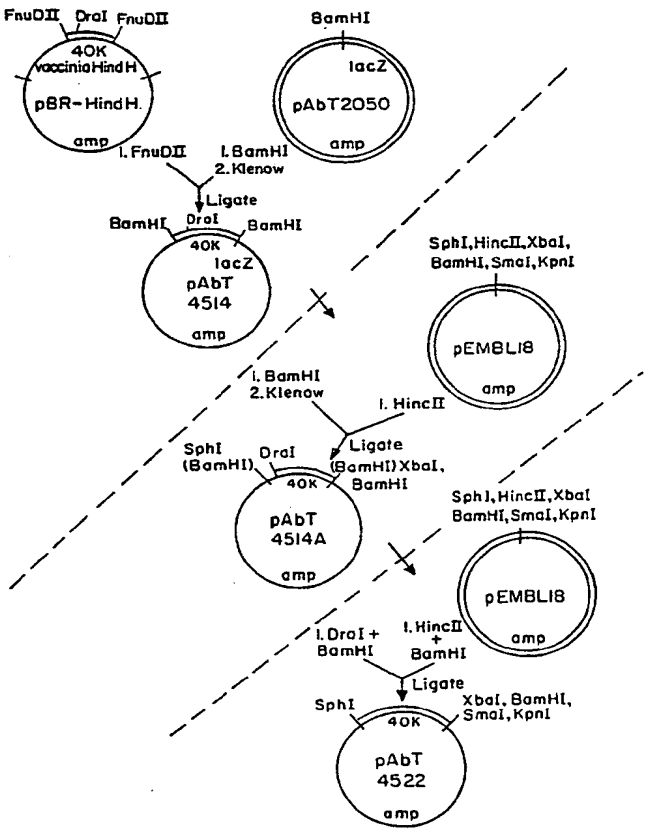
(54) Title: A METHOD OF EVALUATING RECOMBINANT VACCINES AGAINST IMMUNODEFICIENCY VIRUS

(57) Abstract

This invention relates to recombinant pox viruses capable of expressing simian immunodeficiency viral antigens and eliciting protective immune response against SIV. The invention further pertains to a non-human primate model system using macaque monkeys, that serves to evaluate the effectiveness with which recombinant pox virus expressing SIV antigens can protect macaques against acquiring and developing simian AIDS. The importance of the non-human primate model system and methods for generating recombinant pox viruses containing SIV genes and their HIV counterparts lies in the development of recombinant vaccinia viruses expressing a combination of HIV-1 genes or epitopes which will afford total, long term protection against HIV-1 infection in humans. Use of the animal model system of the present invention for vaccine evaluation will enable vaccines to be prepared for use in human trails.



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A METHOD OF EVALUATING RECOMBINANT VACCINES AGAINST IMMUNODEFICIENCY VIRUS

Background

The emergence of the Acquired Immunodeficiency Disease (AIDS) pandemic may represent the most serious public health threat of the twentieth century. Since the recognition of AIDS in 1981, extensive research has resulted in substantial advances in the understanding of the disease. The causative virus, Human Immunodeficiency Virus (HIV), has been identified and the major routes of transmission, at least in the United States, have been shown to be sexual contact and exchange of blood products. The nucleotide sequences of the genomes of many isolates of HIV have been determined and the molecular biology of the virus is under intensive investigation. However, much work remains to be done in elucidating viral replication in infected individuals and its role in the pathogenesis of the disease.

HIV exhibits a remarkable ability to persist and eventually induce chronic disease in spite of host immune defenses. Thus, unlike vaccines that are effective in preventing clinical disease but do not induce absolute resistance to infection, an effective vaccine against AIDS may have to prevent totally the establishment of any foci of infected cells in the human host. This goal is made especially difficult by the great genetic variability of

HIV, particularly in the gene that codes for the surface glycoproteins present in the outer membrane of the virus.

Ideally, an effective vaccine will need to stimulate both humoral and cell-mediated immunity. There are several compelling reasons to believe that cell-mediated immunity may be particularly important in protection against HIV. Cytotoxic T-Lymphocytes (CTLs) can recognize "conserved" regions of viral proteins expressed on the surface of infected cells, including non-envelope viral proteins that are not displayed on the surface of the mature virion particles. Unlike the viral envelope glycoprotein which is highly variable antigenically from isolate to isolate, these other viral proteins show a constancy in their structure and associated antigens. Because the high antigenic variability in the envelope glycoprotein can substantially reduce the efficacy of antibody-mediated protection, which confronts largely this glycoprotein, CTL-mediated functions may be necessary to obtain broad-spectrum resistance to the many HIV variants. Furthermore, the ability of both CTL and NK (natural killer cells) to eliminate virus-infected cells may curtail the spread of infection by preventing cell-to-cell transmission and the resulting propagation of HIV infection. It is important, therefore, to optimize the probability of eliciting protective immunity by developing vaccines with the potential of stimulating both antibody and cell-mediated responses.

Conventional approaches to vaccine development rely upon the use of live, attenuated or killed forms of the virus as immunogens. The success of these approaches in the past was due in part to the presentation of native antigens and the ability of attenuated virus to elicit the complete range of immune responses obtained in natural infection. There are some obvious difficulties with the use of whole virus for an HIV vaccine. Production of such a vaccine would involve significant biosafety problems, as it would require the generation of large quantities of a potentially highly pathogenic virus. Difficulties in vaccine production are not, however, the only consideration. The fear that an attenuated virus could revert to virulence and the danger of incomplete inactivation of killed virus preparations, together with the well-founded reluctance to introduce the entire HIV genome into seronegative individuals, may preclude consideration of live attenuated or killed HIV vaccines for the prevention of infection.

Recombinant DNA technology has made possible the development of vaccines based on the use of defined antigens, rather that the intact infectious agent, as immunogens. These include peptide vaccines, consisting of chemically synthesized, immunoreactive epitopes; subunit vaccines, produced by expression of viral proteins in recombinant heterologous cells; and the use of live viral vectors for the presentation of one or a number of defined antigens.

Both peptide and subunit vaccines are subject to a number of potential limitations. A major problem is the difficulty of ensuring that the conformation of the engineered proteins mimics that of the antigens in their natural environment. Suitable adjuvants and, in the case of peptides, carrier proteins, must be used to boost the immune response. In addition, peptide and subunit vaccines elicit primarily humoral responses and thus may fail to evoke effective cell-mediated immunity.

Infection of susceptible cells with recombinant pox viruses that express the HIV envelope glycoprotein, resulted in the normal synthesis, glycosylation, processing, and membrane transport of the envelope polypeptide, and the polypeptide was recognized by serum antibodies (Hu, et al. (1986) Nature 320:537-540; Chakrabarti et al. (1986) Nature 320:535-537; Kieny et al. (1986) Biotechnology 4:790-794) and by cytotoxic T cells from patients with AIDS (Walker et al. (1987) Nature 328:345-348). The gene product has been shown to be functional by virtue of its ability to induce CD4-dependent cell fusion leading to cell death, one of the characteristic manifestations of HIV cytopathology (Lifson et al. (1986) Nature 323:725-728; Papovic et al. (1984) Science 224:497-500). Vaccination of animals of several species with these recombinants elicited strain-specific humoral immune responses as well as cell-mediated responses (Hu et al. (1986) Nature 320:537-540; Chakrabarti et al. (1986) Nature 320:535-537; Zarling et al. (1986) Nature 323:

344-346; Hu et al. (1987) Nature 328:721-724; Zagury et al. (1987) Nature 326:249-250; Zagury et al. (1988) Nature 332:728-731). Despite these immune responses, however, these vaccines failed to protect chimpanzees from infection by HIV (Hu et al. (1987) Nature 328:721-724). The failure of these initial vaccinia-based HIV vaccines to protect against infection in the chimpanzee system may be attributed to a variety of factors. Certainly, the fact that these vaccines induce expression of only a single HIV protein suggests that they may not have maximum potential for stimulating protective immune responses. The absence of additional HIV antigens in these vaccines may also contribute to their inability to elicit immune responses effective against multiple HIV strains; indeed, the single envelope gene expressed by these recombinant vaccinia vaccines induced primarily strain-specific humoral responses.

The abovementioned analysis of HIV vaccine efficacy in chimpanzees also raises additional problems. Chimpanzees are the only nonhuman primate infectable with HIV and thus constitute the only vaccine-challenge system that has been utilized to date. However, although chimpanzees become infected following HIV inoculations, they do not develop any AIDS-like illness. Consequently, their response to a vaccine many not be predictive of the vaccine's effect in humans. Furthermore, and most importantly, their very limited numbers will severely restrict their further use for such studies.

Testing of numerous vaccines and the variables associated with each will simply not be possible in chimpanzees. These considerations emphasize the importance of developing a more suitable animal model system for vaccine evaluation.

The existence of a closely related virus, called SIV (simian immunodeficiency virus), from macaque monkeys is of interest in this regard. Several isolates of SIV have been cloned and sequenced. The results reveal 40-55% overall identity in the predicted amino acid sequences when compared to HIV-1 and about 75% when compared to HIV-2. Experimental inoculation of this virus into macaque monkeys has consistently resulted in long-term persistent infection, with most inoculated animals dying of a disease remarkable similar to AIDS in humans. There is therefore, a close relatedness of SIV to HIV.

Disclosure of the Invention

This invention pertains to recombinant pox viruses capable of expressing simian immunodeficiency virus antigens, particularly antigens capable of eliciting a protective immune response against simian immunodeficiency virus (SIV) and to methods of producing the recombinant pox virus. This invention also pertains to intermediate DNA vectors which recombine with pox virus *in vivo* to produce the modified pox viruses, to methods of vaccinating a non-human primate host with the recombinant pox virus to elicit protective immunity

against SIV in the host, and to use of non-human primates as a model system to evaluate the effectiveness of pox virus vaccines containing SIV antigens.

Finally, this invention pertains to the application of the information obtained using the SIV/non-human primate model system in the development of pox virus-based vaccines for the prevention of human immunodeficiency virus (HIV) infection of humans. Because of the extensive genetic and biological similarities between HIV and SIV, the data derived from the in vivo evaluation of pox virus vaccines that contain SIV antigens will be applied to the design of pox virus vaccines expressing HIV antigens. This approach will permit the formulation of HIV vaccines which have the maximum potential for efficacy. Thus, if a combination of SIV antigens contained in a pox virus vaccine successfully prevents SIV infection of non-human primates, it is likely that a corresponding pox virus vaccine that contains, in the same combinations, the HIV counterparts of those SIV antigens will effectively prevent HIV infection and the development of AIDS in humans.

Recombinant pox viruses capable of expressing SIV antigens are produced by integrating into the pox virus genome a gene or genes encoding the SIV antigen(s) of interest. The SIV gene(s) is inserted into a region of the pox virus genome which is nonessential for replication of the pox virus. Each gene is inserted into the pox virus genome in

association with a pox virus promoter to direct its expression.

The SIV gene(s) is integrated into the pox viral genome by an in vivo recombination event between the pox virus and an intermediate DNA vector carrying the SIV gene. In essence, the intermediate DNA vector contains the SIV gene linked to a pox viral promoter located within a DNA sequence homologous to a region of the pox viral genome which is nonessential for replication of the pox virus. The intermediate vector must also have the capacity to replicate in a prokaryotic host; amplification of the vector in a prokaryotic host cell allows for the production of necessary amounts of vector DNA for in vivo recombination. Furthermore, in order to detect the presence of the intermediate plasmid vector in the prokaryotic host, the vector must also contain a gene which confers a selectable phenotypic trait on the prokaryotic host cell. Thus, the vector comprises:

a. a prokaryotic origin of replication;

b. a gene encoding a marker for selection in the prokaryotic host;

c. a pox viral promoter;

d. a gene encoding a simian immunodeficiency virus antigen under the direction of the pox viral promoter;

e. DNA sequences of the pox virus into which the gene encoding the SIV antigen is to be integrated. These DNA sequences flank the promoter and structural gene at both the 5' and 3' ends and are

homologous to the region of the pox virus genome where the SIV gene is to be inserted.

Recombination of the DNA vector and the pox virus is achieved in an appropriate host cell. Appropriate host cells for in vivo recombination are eukaryotic cells which are 1) transfectable by the DNA vector and 2) infectable by pox virus. The host cell is infected with the pox virus and then is transfected with the DNA vector. Virus is allowed to replicate in the host cell and recombination occurs in vivo, resulting in insertion of the SIV gene into the pox virus genome.

The viral progeny is isolated away from the wild type virus. When a selectable marker has been co-integrated with the SIV antigen, expression of the selectable marker provides a basis for selection of recombinant virus containing integrated SIV DNA. Other methods of selection include detection of integrated SIV gene by hybridization with homologous DNA probes or selection for absence of the product of the viral gene into which the DNA vector has been inserted.

The recombinant virus is a virus which expresses in tissue culture and in an inoculated animal host the SIV antigen(s) of interest. The antigen expressed by the recombinant pox virus will elicit cell-mediated and humoral responses which may be capable of recognizing and neutralizing the SIV from which the antigen is derived. Moreover, certain antigens which are shared antigenic

determinants may provide cross-protection against the various simian viruses.

In preferred form, the DNA vector for recombination with pox virus also contains a gene encoding a selectable marker which permits selection of viral recombinants containing the inserted SIV DNA. Thus, the vector will contain these additional elements located between the flanking pox viral sequences:

f. a second pox viral promoter; and

g. a gene encoding a selectable marker, the gene being under the direction of the second pox viral promoter.

This invention also pertains to methods of using a simian model to evaluate the effectiveness with which recombinant pox viruses expressing SIV antigens protect monkeys from acquiring an SIV infection and developing Simian AIDS, in order to identify HIV homologs effective in treating or preventing AIDS in humans. The preferable monkey to be used in this model system is the macaque monkey. SIV pox-viral recombinants are tested for their ability to protect macaques from SIV infection. The extremely close correspondence between SIV and HIV genomic material makes it likely that information obtained from work on the simian model system will be applicable to the design of a safe and effective pox virus-based HIV vaccines for human use. Therefore, this invention also pertains to the development of DNA vectors and pox virus containing HIV counterparts of SIV DNA sequences.

There are a number of advantages to creating a recombinant vaccinia virus expressing HIV genes for use as a live vaccine. There is a long history of the successful and safe use of vaccinia virus for the eradication of smallpox. Vaccination with a live virus will stimulate cell-mediated and humoral immunity. The proteins that are expressed are expected to be appropriately modified, and if the required signals are present, they may also be localized to the proper regions of the cell or cellular membrane (Stephans et al.(1986) EMBO J. 5:237). In addition, it has been shown that at least 25Kb of foreign DNA can be inserted into vaccinia virus (Smith and Moss (1983) Gene 25:21). This amount of DNA can include a number of foreign genes encoding various antigens. Vaccinia virus has been shown to be stable upon storage under normal field conditions. The vaccine can also be administered relatively simply. Finally, a recombinant vaccine utilizing vaccinia virus would avoid the problems associated with vaccinating with live attenuated or killed virus, namely the possibility that vaccines vased on whole viral pathogens may not be properly killed or may revert to virulence. It is expected that live viral vaccines would also be more immunogenic than subunit vaccines.

Brief Description of the Figures

Figure 1 shows the similar genomic organization of Simian Immunodeficiency Virus (SIV) and Human Immunodeficiency Virus (HIV-1).

Figure 2 shows the construction of pAbT4500 and pAbT4525, plasmid vectors for insertion of foreign genes into vaccinia by in vivo recombination (IVR) containing the 30k or 40k promoter, respectively, the vaccinia thymidine kinase gene region for directing the recombination, a lacZ gene under the control of the vaccinia BamF promoter, and a bacterial replicon and ampicillin resistance gene.

Figure 3 shows the construction of pAbT4537, identical to pAbT4525 except that the thymidine kinase region has been shortened and modified to contain additional, convenient restriction sites.

Figure 4 shows the construction of IVR vector pAbT4554, which contains the vaccinia HindIIIM region for directing recombination, the 29k host range gene for selection of recombinants, the vaccinia 30k promoter and multiple cloning site for insertion of foreign genes, and a bacterial replicon and ampicillin resistance gene.

Figure 5 shows the construction of IVR vectors pAbT4555 and pAbT4556, which are identical to pAbT4554, except that pAbT4555 contains two promoters, 30k and 40k, and pAbT4556 contains three promoters, 30k, 40k and 7.5k, each followed by a multiple cloning site for the insertion of foreign genes.

Figure 6 shows the construction of IVR vector pAbT4586, which is identical to pAbt4537, except that pAbT4586 contains two vaccinia promoters, 7.5k and 40k, each followed by a multiple cloning site for the insertion of foreign genes.

Figure 7 shows the construction of plasmid pAbT4572, a plasmid vector for the insertion of the $SIV_{MAC-251}$ env gene into vaccinia, with the env gene under the control of the vaccinia 40k promoter.

Figure 8 shows the construction of plasmids pAbT4575 and pAbT 4577. pAbT4575 is an IVR vector for the insertion of the $SIV_{MAC-251}$ gag-prot gene into vaccinia with the gag-prot gene under the control of the 40k promoter. pAbT4577 is an IVR vector for the insertion of the $SIV_{MAC-251}$ env and gag-prot genes into vaccinia, with the env gene under the control of the 30k promoter and the gag-prot gene under the control of the 40k promoter.

Figure 9 shows the construction of plasmid pAbT4576, an IVR vector for the insertion of the $SIV_{MAC-251}$ 3'-orf gene into vaccinia, with the 3'-orf gene under the control of the 30k promoter.

Figure 10 shows the construction of plasmid pAbT4581, an IVR vector for the insertion of the $SIV_{MAC-251}$ sor gene into vaccinia, with the sor gene under the control of the 30k promoter.

Figure 11 shows the construction of plasmid pAbT4583, an IVR vector for the insertion of the $SIV_{MAC-251}$ pol gene into vaccinia, with the pol gene, modified by the addition of a translation initiation codon ATG, under the control of the 40K promoter.

Figure 12 shows the construction of plasmid pAbT4585, an IVR vector for the insertion of the $SIV_{MAC-251}$ env and gag-prot genes into vaccinia, with the env gene under the control of the 40k

promoter and the gag-prot gene under the control of the 7.5k promoter.

Figure 13 shows the construction of plasmid pAbT4589, an IVR vector for the insertion of the $SIV_{MAC-251}$ env and gag-prot genes into vaccinia. pAbT4589 is identical to pAbT4585, except that pAbT4589 contains elements for insertion at the vaccinia thymidine kinase region, whereas pAbT4585 contains elements for insertion and selection at the vaccinia HindIIIM region.

Detailed Description of the Invention

1. Genes Encoding SIV Antigens

Genes encoding simian immunodeficiency viral antigens can be obtained from the genomic cDNA of a simian immunodeficiency virus or from available subgenomic clones containing the genes. The genomic organization of both the HIV and SIV genomes is represented diagrammatically in Figure 1. The gene organization of the two viruses is remarkably similar. Homologs of each of the HIV-1 open reading frames are present in SIV. Because the structure of SIV is extremely similar to that of HIV, the assumption has been made that data from HIV can be extrapolated to SIV.

The HIV virion is composed of a core surrounded by a lipid membrane containing the envelope (env) glycoprotein. This bipartite envelope protein has been considered the most promising candidate on which to base a vaccine strategy. The envelope glycoprotein is believed to play a key role in

HIV-induced disease. It specifically reacts with the CD4+ surface antigen to mediate both virus-to-cell and cell-to-cell transmission and, in addition, mediates a fusion reaction between infected cells which express the glycoprotein on the surface and CD4+ cells. This reaction, which is postulated to result in the formation of inviable, multinucleated syncytia as well as in the death of individual cells, may account in part for the progressive nature of HIV and possibly SIV disease.

The core particle of HIV, which contains the viral RNA, as well as reverse transcriptase and integrase proteins, is surrounded by a protein shell or capsid. The capsid proteins, which are more highly conserved among strains than are the envelope proteins, are derived from the viral gag gene. These proteins are primarily located beneath the lipid envelope. The presence in infected patients of antibodies to the gag proteins of HIV may correlate with the stage of disease, with more seriously ill individuals exhibiting little or no anti-gag reactivity. The gag proteins could serve as an important immunogens in a recombinant vaccine.

Other genes encoded by the HIV virus, such as pol and sor, may also encode important immunogens appropriate for inclusion in a recombinant vaccine. The pol gene, like gag is well conserved among different isolates and encodes a polypeptide which is proteolytically cleaved into a protease, a reverse transcriptase, and an endonuclease. Antibodies to the HIV-1 endonuclease and reverse

transcriptase have been detected in infected individuals. In addition, CTL specific for HIV-1 reverse transcriptase have been detected in HIV-1 infected individuals (Walker et al. (1988) Science 240:64-66).

The HIV-1 sor gene product is required for cell-free viral infection of cells. HIV strains in which the sor gene has been deleted are defective in their ability to transmit in a cell-free manner. Antibodies to the sor gene product have been detected in infected individuals; these antibodies could play a role in preventing infection of cells by HIV and, possibly, SIV viral products.

HIV and SIV also contain at least four additional genes. The tat gene encodes a protein that functions as a potent trans-activator of HIV gene expression. The art gene product also up-regulates HIV synthesis by a transacting antirepression mechanism. In contrast, the 3'-orf gene product may down-regulate virus expression. Finally, the R gene codes for an immunogenic protein whose function is currently unknown.

The SIV and HIV genes selected for expression in recombinant pox viruses of this invention, and their protein products, are outlined in Table 1. The three major virion components derived from the env, gag, pol and sor genes are synthesized as precursor polyproteins which are subsequently cleaved to yield mature polypeptides outline in Table 1.

Table 1. Selected SIV and HIV Genes and Gene Products for Recombination into Vaccinia Virus

Gene	Gene Product[a]		Processed Peptides[a]
env	gp160	gp120	extracellular membrane protein
		gp41	transmembrane protein
gag	p55	p24	
		p17	capsid proteins
		p15	
pol	p160(b)	p10	protease
		p51/p66	reverse transcriptase
		p31	endonuclease
sor	p23	p23	
3'orf	p27	p27	

a. Sizes given are for HIV proteins; sizes of the SIV polypeptides may be slightly different

(b) part of gag-pol product

The env and sor genes, can be expressed in pox virus by insertion of the relevant coding region into the viruses under the direction of pox viral promoters. However, the expression of gag and pol products is more complicated. In HIV and SIV the gag-pol domain is apparently transcribed into a single mRNA that encodes two polypeptides: the gag precursor polypeptide (p55), which is processed by the virus-encoded protease to yield the mature capsid

proteins; and a gag-pol fusion polypeptide, encoded in the -1 reading frame with reference to gag, which is processed to yield a protease, reverse transcriptase, and endonuclease. Synthesis of the gag-pol fusion polypeptide occurs as a result of ribosomal framshifting; typically, retroviral gag and gag-pol precursor polypeptides are synthesized at a ratio of 10-20 to 1.

In view of the complexity of the gag-pol domain, several strategies exist for the expression of SIV or HIV gag and pol proteins by recombinant pox viruses. A recombinant pox virus can be created that contains the entire SIV or HIV gag-pol domain under the control of a single pox virus promoter. The pattern of gag and pol expression in this recombinant will likely depend upon the efficiency of ribsomal frameshifting; if it is highly inefficient, the predominant protein product will be unprocessed gag precursor polypeptide.

In order to express high levels of processed gag and pol proteins a pox viral recombinant can be constructed in which separate promoters are used to direct the expression of the SIV gag and pol genes. To do this, site directed mutagenesis can be used to modify the 5' end of the pol gene in order to permit its individual expression. Recombinant pox viruses that express either SIV gag or pol are constructed. A single recombinant that expresses both of these genes under the control of separate promoters can be constructed.

2. Pox Viruses

A suitable pox virus for generating recombinant viruses includes any member of the pox family which does not cause significant disease in normal humans or animals. The preferred pox virus is vaccinia virus, a relatively benign virus which has been used for years as a vaccine against smallpox. Vaccinia virus is capable of infecting non-human primates such as monkeys and thus can be evaluated in the model system of this invention. General techniques for integration of heterologous DNA into vaccinia virus to provide modified vaccinia virus capable of expressing foreign protein encoded by the heterologous integrated DNA are described by Paoletti et al., U.S. Patent No. 4,603,112, the teachings of which are incorporated by reference herein.

3. Preparation of intermediate DNA vectors for in vivo recombination with pox virus

According to the method of this invention cloned SIV genes or their HIV counterpart genes which code for antigenic proteins are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by the pox virus along with the expression of the normal complement of pox virus proteins. This is accomplished by first constructing a prokaryotic insertion vector.

This vector is capable of carrying either SIV genes or the HIV counterparts of the SIV genes (e.g., gag, pol, env, sor, 3'orf, art, tat). In the following description of intermediate DNA vectors

suitable for recombination with pox virus, foreign DNA sequences to be inserted into the vector are those of the SIV genome. It is understood that corresponding DNA sequences of the HIV genome will also be suitable.

The vector contains (i) a prokaryotic origin of replication so that the vector may be amplified in a prokaryotic host, (ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance) (iii) DNA sequences homologous to the region of the pox virus genome where SIV gene is to be inserted, (iv) one or more SIV genes inserted at a site within this pox virus sequence and (v) one or more pox virus regulatory sequences (promoters) adjacent to the 5' end of each SIV gene, constructed in a manner to allow for transcription of the SIV gene and subsequent expression. All pox virus DNA fragments, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the pox virus genome into which foreign DNA is to be inserted, are obtained from genomic DNA or cloned DNA fragments. The SIV genes are obtained as described above. The vector backbone can be derived from any of several plasmid vectors capable of replication in a prokaryotic host, e.g., pBR322 (Sutcliffe, Cold Spring Harbor Symp., Quant. Biol., 43:77 (1979), pUC8 (Vieira and Messing, Gene, 19:259 (1982) or pEMBL (Denta et al., Nucleic Acids Res., 11:1645 (1982)).

The ability to replicate in a prokaryotic host provides a means for amplification of the vector to produce sufficient quantities for transfection of a eukaryotic cell for recombination.

In addition, the vector preferably contains a gene which encodes a marker which will allow selection of recombinant pox viruses containing integrated SIV DNA. The gene encoding the marker is placed under control of a pox virus promoter. Several types of marker genes can be used. A preferred marker gene is the E. coli lacZ gene which encodes the enzyme beta-galactosidase. Recombinant pox virus will express beta-galactosidase along with the SIV antigen. Thus, beta-galactosidase production is detected as an indication of insertion and expression of the foreign DNA. In the presence of a chromogenic indicator (BluoGal) recombinant viruses which express beta-galactosidase will form blue plaques while wild-type virus, which do not express beta-galactosidase will form clear plaques. Furthermore, a recombinant virus that has sequences inserted into the pox virus TK (thymidine kinase) gene will have an inactivated TK gene and will grow in the presence of BUdR, while virus containing a wild-type, uninterrupted TK gene will be unable to grow in the presence of BUdR. Dubbs and Kit (1964) Virol., 22:214; Smith et al. (1983) Proc. Natl. Acad. Sci., USA, 80:7155. Other selectable markers include genes which confer antibiotic resistance in an infected host cell, e.g., the Neo^R gene. Infected cells are grown in media containing the

antibiotic at a concentration toxic for antibiotic sensitive cells. Under these conditions, cells infected with a recombinant virus expressing the resistance marker will produce virus. Cells infected with virus not containing the marker will not produce virus.

Another selection procedure relies upon a single vaccinia-encoded function. This procedure obviates the need for the use of drugs, mutagens, or chromogenic indicators, and permits rapid (2-3 wk) purification of recombinant viruses. The method is described in co-pending U.S. patent application Serial No. 205,189 , entitled "Method Of Selecting For Recombinant Pox Viruses", Attorney's docket number ABT88-02, filed concurrently with this application, the teachings of which are incorporated herein by reference, Briefly, a vaccinia virus is employed that contains a mutation in a specific structural gene (29k gene), located in the HindIIIM fragment of vaccinia virus. This mutation prevents the growth of the virus on particular host cells, for example RK13 (rabbit kidney) cells. The intermediate DNA vector contains vaccinia DNA sequences capable of restoring the mutant gene function; these sequences also direct recombination to the site of the mutant gene in the HindIIIM region. Thus, recombinant vaccinia viruses regain the ability to grow on RK13 cells, and can be isolated from non-recombinant viruses, which are unable to grow on these cells.

A preferred DNA vector for recombination with the preferred vaccinia virus comprises:

a. one or more vaccinia promoters (e.g., the vaccinia 11K, 7.5K, 30K, 40K or BamF promoter or modified versions of these promoters), each linked to;

b. one or more structural genes encoding SIV antigens of interest (e.g., the gag, env, pol, sor, 3'orf antigen) each under control of a promoter;

c. a marker for the selection of recombinant vaccinia virus, which may comprise:

(1) a vaccinia promoter (e.g., the BamF promoter of vaccinia virus linked to a gene encoding a selectable marker (e.g., the E. coli lacZ gene; or

(2) vaccinia structural gene sequences which restore the function of the 29k polypeptide;

e. DNA sequences homologous with a region of vaccinia nonessential for replication flanking the construct of elements a-d (e.g., the vaccinia TK or HindIIIM sequence).

4. In vivo recombination

The intermediate DNA vectors containing the SIV gene(s) and the marker gene flanked by appropriate pox viral sequences undergo recombination with pox virus genomic DNA, which results in integration of

the flanked gene into the viral genome. Recombination occurs in a eukaryotic host cell. Appropriate host cells for recombination are those which are 1) infectable by pox virus and 2) transfectable by the DNA vector. Examples of such cells are chick embryo fibroblast, CV-1 (monkey kidney cells), HuTK 143 cells (human cells), BSC40 (monkey kidney cells), RK-13 cells (rabbit kidney cells) and many others.

Viral infection is accomplished by standard techniques for infection of eukaryotic cells with pox virus.

The cells can be transfected with the intermediate vector by any of the conventional techniques of transfection. These include the technique of calcium phosphate precipitation, DEAE dextran, electroporation and protoplast fusion. The preferred technique is the calcium phosphate precipitation technique.

After infection and subsequent transfection, the cells are incubated under standard conditions and virus is allowed to replicate, during which time in vivo recombination occurs between the homologous pox virus sequences in the intermediate vector and the pox virus sequences in the genome.

Recombinant viral progeny are then identified by any of several techniques. For example, virus harboring the SIV gene(s) can be selected on the basis of inactivation of the viral gene into which foreign DNA was inserted. For example, if the DNA vector is designed for insertion into the TK gene,

viruses containing integrated DNA will be TK^- and can be selected on this basis. Preferred techniques for selection, however, are based upon co-integration of a gene encoding a marker or indicator gene as described above. One preferred indicator gene is the E. coli lacZ gene. Selection of recombinant viruses expressing B-galactosidase can be done by employing a chromogenic substrate for the enzyme. A second preferred indicator gene is the vaccinia 29K gene. Selection of recombinant viruses that express the wild-type 29K gene-encoded function can be performed by growing the recombinant virus on RK-13 cells.

5. Characterizing the expression of SIV antigens by the recombinant viruses.

Once a recombinant vaccinia virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an in situ enzyme immunoassay performed on viral plaques), Western blot analyses, radioimmunoprecipitation (RIPA), immunofluorescence assays (IFA), and enzyme immunoassays (EIA). The antibody used for these analyses is sera from macaque monkeys infected with SIV.

Recombinant viruses that contain genes encoding the envelope proteins of SIV can also be assayed for their ability to bind the CD4 receptor and induce the formation of syncytia in vitro. Syncytia formation mimics, at least qualitatively, the

processes involved in SIV entry into the host cell and can be used as a model to test the ability of an envelope polypeptide produced by vaccinia virus to function in this regard.

6. Testing Responses to SIV Antigens Expressed by Recombinant Vaccinia Viruses

Recombinant vaccinia viruses that express SIV antigens can be initially evaluated in mice to determine the nature and magnitude of the humoral immune responses they elicit. Sera are assayed for the presence of antibodies specific for the expressed antigens and for the ability to neutralize SIV in vitro.

Briefly, mice are inoculated with 10^8 pfu of recombinant virus by intraperitoneal injection. Serum samples obtained weekly for 4-6 weeks are assayed for anti-vaccinia titers using an enzyme-linked immunoabsorbant assay (ELISA). The sera are then examined for specific response to the expressed antigens using the following assays:

1. ELISA. For SIV recombinants, sera will be tested for SIV-specific humoral immune responses using a whole virus ELISA. For this assay, SIV purified from infected cells using a column chromotography procedure is used to coat the wells of microtiter plates, and the ELISA is performed using serial dilutions of the mouse sera.

2. Western Blot Analaysis. To examine the antibody responses to SIV polypeptides, a prepara-

tion of SIV virus can be electrophoresed through SDS-polyacrylamide gels and Western blots prepared using conventional procedures. Maniatis, T. et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, (1982).

3. Radioimmunoprecipitation (RIPA). RIPA can also be used to detect SIV-specific humoral immune responses. Radiolabelled whole SIV virus or SIV polypeptides expressed in heterologous mammalian expression systems can be used as the source of antigen for these studies.

A parameter believed to be critical for the ability of vaccine-induced antibodies to protect against virus challenge is the ability of the antibodies to neutralize virus infectivity. Several methods are used to evaluate the neutralization capabilities of antibodies elicited by the vaccinia recombinants:

a) Inhibition of syncytia formation. The ability of an antiserum to inhibit the formation of syncytia correlates with its ability to neutralize HIV virus infection. Fung et al. (1987) Biotechnology 5:940-946. Simian immunodeficiency virus is incubated with heat-activated murine sera, then inoculated onto c8166 (CD4+) cells. The presence of syncytia is scored at day 4 and at day 7. Weiss et al. (1986) Nature 324:572-575.

b) In situ hybridization neutralization assay. In this assay, SIV is incubated with heat-inactivated murine sera, then inoculated onto CEM cells in a

24-well plate. After 5 days in culture, the cells are removed from the wells and adhered to glass slides. In situ hybridization using SIV cDNA and a nonisotopic detection method is used to quantitate virally infected cells. Byron et al., III International Conference on AIDS, 163 (abstract).

3. ^{3}H-Thymidine incorporation assay. To assay the neutralization titers of mice inoculated with SIV/vaccinia recombinants, an assay is used that is particularly sensitive and well-suited to analysis of multiple samples. It depends on the extreme sensitivity of MT-4 cells to cytopathic effects and on the ability of antisera with neutralizing activity to prevent these cytopathic effects. SIV virus is incubated with the murine sera, then added to MT-4 cells growing in 96-well plates. Infected cells are killed and do not incorporate ^{3}H-thymidine while cells protected from SIV infection do incorporate ^{3}H-thymidine. Daniel et al., (1988) Int. J. Cancer 41:601.

Based on the analysis of the immune responses, vaccinia recombinants that express one or more SIV antigens and that elicit good levels of neutralizing antibodies in mice will be selected for detailed testing in macaque monkeys.

6. Vaccine Efficacy Studies in the SIV/Macaque Model System

Testing of recombinant pox virus containing SIV DNA sequences encoding immunogenic SIV proteins includes measurement of total antibody response, of

neutralizing antibody titers, of cell-mediated responses, and of the response of macaques vaccinated with the SIV/vaccinia recombinants to live SIV challenge.

General Methods

Measurement of total antibody response. The total antibody response in monkeys inoculated with recombinant vaccinia viruses expressing SIV antigens will be measured using a whole virus enzyme, linked immunosorbant assay (ELISA). SIV is purified from infected cells using a column chromatography procedure and ELISA plates are coated with whole lysed virus. Antibodies in the same vaccinated monkey at different times and in different monkeys receiving the same vaccinia recombinant under different conditions (e.g., different dose, route, number of injections) can be compared in titer by their reaction in the whole virus ELISA.

Identification of recognized antigens. Both Western blot analysis and radioimmunoprecipitation (RIPA) can be used to identify the specific SIV antigen(s) recognized by antibodies from vaccinated macaques, both before and after challenge with live SIV.

Neutralization of virus infectivity. The ability of vaccine-induced antibodies to neutralize viral infectivity is believed to be a parameter critical for protection against virus challenge. A number of neutralization assays for SIV are currently used, including the ^{3}H-thymidine incorporation assay previously described.

Analysis of cell-mediated immunity. SIV-specific proliferative responses, suppression of SIV outgrowth by CD8+ lymphocytes, and the nature and magnitude of cytotoxic T-lymphocyte responses can be measured.

Recovery of SIV from challenged monkeys. Samples of heparinized peripheral blood can be obtained from SIV-inoculated monkeys at periodic intervals. The ability to recover SIV from the adherent cell population and from the PHA-stimulated lymphocytes by co-cultivation with indicator cells can be monitored by the appearance of reverse transcriptase activity in the cell culture supernatant.

Design of SIV Challenge and Vaccination Procedures in Macaques

The design of vaccination and challenge experiments is illustrated by the evaluation of vaccinia recombinants that express SIV env and gag polypeptides, in monovalent and divalent conformations. The recombinants to be analyzed can be those that have expressed high levels of the inserted SIV gene products and that have elicited good anti-SIV immune responses in mice.

1. Macaque Monkeys

Juvenile rhesus macaques (Macaca mulatta) are chosen for inoculation with each vaccinia recombinant. Two age-matched rhesus macaques can serve as unvaccinated controls. The macaques can be pre-screened for good health, normal blood counts, and absence of anti-SIV antibodies.

2. Vaccination

In an attempt to maximize immune response in macaques, both the dose of recombinant vaccinia virus administered and the number of inoculations can be examined. For evaluation of vaccinia recombinants expressing SIV env and gag genes, the vaccination scheme can be as follows:

SIV gene expressed	Number of macaques
env	2
gag	2
env + gag	2
unvaccinated controls	2

One macaque in each group can be inoculated with 10^8 PFU intradermally while the other can receive 10^9 PFU. Blood samples can be taken at weeks 2 and 4 and vaccination with the same dose at a different site can be repeated at week 4. Vaccination can be continued every four weeks until such time that anti-SIV antibody titers are no longer increased.

3. Immune Responses

Humoral and cell-mediated immune responses measurements can be made as described in the previous section. These measurements are important for two reasons: 1) they provide preliminary evidence for the adequacy of the immune response; and 2) they serve to eventually identify the immune response parameters that correlate with protection.

4. Live SIV Challenge

Shortly after the last vaccination, vaccinated and control macaques can be challenged with a minimal dose of previously titered, cell-free, live, pathogenic SIV. It is important to use a minimal dose of cell-free virus because of the need to optimize the chance of successful protection in the initial experiments. Various dilutions of the frozen stock SIV to be used for these experiments will have been inoculated previously into macaques; 1 ml of a 10^{-4} dilution of this pathogenic stock virus (SIVmac251) can be administered intramuscularly for our initial challenges. (Clones derived from the SIVmac251 virus were used for the construction of the vaccinia recombinants).

5. Vaccine Evaluation

Two main criteria can be used to evaluate the success of each vaccine: 1) the ability to recover SIV from peripheral blood of challenged macaques; and 2) disease induction. Heparinized peripheral blood can be obtained every two weeks for the first eight weeks and once per month thereafter. Attempts are made to recover SIV from the adherent (monocyte/ macrophage) cells and the non-adherent, stimulated lymphocyte population by co-cultivation with indicator cells. If SIV recovery attempts are repeatedly negative from vaccinated macaques, an attempt can be made to recover SIV from the peripheral blood following depletion of CD8+ (supressor/ cytotoxic) cells by monoclonal antibody panning. Evidence from SIV recovery are correlated with clinical evidence

and eventual outcome. Greater than 50% of unvaccinated macaques die within 12 months of inoculation with this SIV stock. Failures and partial successes warrant an examination of which antigens should be incorporated into a vaccine strain, alternative routes of vaccination, vaccine dose, and mechanisms of increasing antigen expression or enhancing immune response.

The invention is illustrated further by the following Examples.

MATERIALS AND METHODS

Cells and Virus

E. coli stain MC1061 (Casadaban and Cohen, 1980, J. Mol. Biol. 138, 179) was used as the host for the growth of all plasmids. The monkey kidney cell line BSC-40, the rabbit kidney cell line RK13 (Beale et al., (1963) Lancet, 2,640) and the thymidine kinase - deficient (TK^-) human cell line $Hu143TK^-$ were used for vaccinia virus infections and transfections.

Vaccinia virus strain New York City Board of Health (NYCBH); ATCC #VR-325) and $29K^-$ $lacZ^+$ strain vAbT33 (See, U.S. Patent Application Serial No. 205,189, filed concurrently herewith) were used as the parental virus for in vivo recombination.

Enzymes

Restriction enzymes were obtained from New

England BioLabs or Boehringer-Mannheim. The large fragment of DNA polymerase (Klenow) was obtained from United States Biochemical Corp., T4 DNA polymerase was obtained from New England BioLabs and T4 DNA ligase was obtained from Boehringer-Mannheim.

Molecular Cloning Procedures

Restriction enzyme digestions, purification of DNA fragments and plasmids, treatment of DNA with Klenow, T4 DNA polymerase, ligase or linkers and transformation of E. coli were performed essentially as described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), incorporated herein by reference.

Oligonucleotide mutagenesis was performed using synthetic oligonucleotides obtained from the Biology Department, Brandeis University, with reagents supplied by Amersham and used according to the manufacturer's instructions.

Preparation of Vaccinia Virus Recombinants

Viral infection, transfections, plaque purification and virus amplification were performed essentially as described (Spyropoulos et al., 1988, J. Virol., 62:1046-1054). TK^- recombinant plaques were selected and purified in the presence of 50 uM bromodeoxyuridine. $29K^+$ recombinants were selected and purified on RK13 cells (see U.S. Patent

Application Serial No. 205,189 , filed concurrently herewith).

Vaccinia Virus Genomic Analysis

DNA was extracted from vaccinia virus-infected cells as described (Esposito et al., J. Virol. Methods, 2:175 (1981)) and analyzed by restriction enzyme digestions and Southern hybridization as described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982). Vaccinia virus recombinant DNA was subcloned into phage m13mp18 (New England BioLabs) and sequenced by the chain-termination method (Sanger et al., Proc. National Acad. Sci. USA, 74:5463 (1977)).

Protein Analysis

Black plaque assay was performed essentially as described in U.S. Patent Application Serial No. 910,501 filed September 23, 1986, using antisera from SIV-infected macaques, obtained from Ronald C. Desrosiers (New England Regional Primate Research Center (NERPRC), Southborough, MA.)

Immunoprecipitation analysis was performed essentially as described in U.S. Patent Application Serial No. 910,501 filed September 23, 1986. Infected cells were labelled either with [^{3}H] leucine or [^{35}S] methionine. Antisera from SIV-infected macaques were used.

Mouse Immunizations

Female Balb/c mice were immunized I.P. with 10^7 - 10^8 pfu vaccinia virus recombinant. Sera were obtained three weeks post-immunization.

Characterization of Mouse Immune Sera

Anti-vaccinia antibody titers of sera from immunized mice were determined by enzyme-linked immunosorbant assay (ELISA), using vaccinia strain WR as antigen, performed essentially as described in U.S. Patent Application Serial No. 910,501, filed September 23, 1986.

Anti-SIV antibody titers of sera from immunized mice were determined by ELISA, using a whole-virus lysate from column-purified SIV. Daniel et al., (1988) Int. J. Cancer 41:601.

SIV neutralization titers of sera from immunized mice were determined by a ^{3}H-thymidine incorporation assay, which depends on the extreme sensitivity of the HTLV-transformed cell line MT-4 to the cytopathic effect of SIV and the ability of antisera with neutralizing activity to prevent these cytopathic effects. SIV virus is incubated with the murine sera, then added to growing MT-4 cells. Infected cells are killed and do not incorporate ^{3}H-thymidine whereas cells protected from SIV infection do incorporate ^{3}H-thymidine. Daniel et al., (1988) Int. J. Cancer 41:601.

EXAMPLE 1: Recombination DNA Vectors Containing Vaccinia Promoters

This Example illustrates construction of monovalent *in vivo* recombination vectors containing the vaccinia 40K or 30K promoter, with insertion at the vaccinia *tk* region (Figures 2, 3). The construction and structure of vectors pAbT4024, pAbT752, pAbT4500, pAG3, pAbT4007, have been described in U.S. Patent application Serial No. 910,501, filed September 23, 1986 incorporated herein by reference.

The vaccinia 40K promoter is located on the 8600bp vaccinia HindIII H fragment (Rosel et al. (1986) *J. Virology*, 60:436-449). A plasmid, pBR-Hind H, containing the HindIII H fragment, was obtained from Bernard Moss (National Institutes of Health, Bethesda, MD). pBR-Hind H was digested with FnuDII, and a 224bp fragment containing the 40K promoter was gel-purified. The cloning vector DNA was an *E. coli* plasmid, derived from pBR322 (Bolivar et al. (1977) *Gene*, 2:95), denoted pAbT2050, the relevant features of which are a unique BamHI site, an *E. coli* replicon and an ampicillin-resistance gene (pAbT2050 also contains a *lacZ* gene but this is not required for the construction). pAbT2050 was digested with BamHI, treated with Klenow, and was ligated to the 224 bp fragment to create pAbT4514, as shown in Figure 2A. pAbT4514 was digested with BamHI, treated with Klenow, and a 230bp fragment containing the 40K promoter was gel-purified. pEMBL18 (Dente et al. (1983) *Nucl. Acids Res.*, 11:1645) was digested with HincII and was ligated to

the 230 bp fragment to create pAbT4514A, as shown in Figure 2B.

pAbT4514A was digested with DraI and BamHI, and a 161bp fragment containing the 40K promoter was gel-purified. pEMBL18 was digested with HincII and BamHI, and was ligated to the 161bp fragment to create pAbT4522, as shown in Figure 2C.

pAbT4024 was digested with SphI and KpnI, and a 420bp fragment containing the vaccinia 30K promoter (Perkus et al., (1985) Science, 229:881) was gel-purified. pAbT752 was digested with SphI and KpnI, and a 7900bp fragment was gel-purified and ligated to the 420bp fragment to create pAbT4500, as shown in Figure 2D.

pAbT4500 is a plasmid vector for use in in vivo recombination experiments in vaccinia. pAbT4500 contains the vaccinia TK region for directing recombination, a lacZ gene under the control of the vaccinia BamF promoter for identification of vaccinia recombinants, the 30K promoter followed by a multiple cloning site for the insertion and expression of foreign antigens, and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

pAbT4522 was digested with SphI and KpnI, and a 170bp fragment containing the 40K promoter was gel-purified. pAbT4500 was digested with SphI and KpnI, and a 7900bp fragment was gel-purified and ligated to the 170bp fragment to create pAbT4525, as shown in Figure 2E.

pAbT4525 is also a plasmid vector for use in in vivo recombination experiments in vaccinia. pAbT4525 is identical to pAbT4500, except that the 40K promoter is substituted for the 30K promoter.

pAbT4525 was slightly modified as follows: pAG3 was digested with NdeI, treated with Klenow, and ligated to NotI linkers (New England BioLabs; dGCGGCCGC) to create pAbT4524A, as shown in Figure 3A. pAbT4524A was digested with NotI, treated with Klenow, and ligated to DraI linkers (Biology Dept., Brandeis University; dGCTTTAAAGC) to create pAbT4524B, as shown in Figure 3B. NotI sites were expected to be regenerated both 5' and 3' to the DraI site; due to exonuclease digestion, the NotI site 3' to the DraI site was not regenerated.

pAbT4007 was digested with DraI, and a 5300bp fragment containing the vaccinia 7.5K promoter, the lacZ gene under the control of the BamF promoter, and most of the tk flanking regions, was gel-purified. pAbT4524B was digested with DraI and was ligated to the 5300bp fragment to create pAbT4532B, as shown in Figure 3C.

pAbT4525 was digested with SphI and KpnI, and the 170bp fragment containing the vaccinia 40K promoter was gel-purified. pAbT4532B was digested with SphI and KpnI, and a 7300bp fragment was gel-purified and ligated to the 170bp fragment to create pAbT4537, as shown in Figure 3D.

pAbT4537 is a plasmid vector for use in IVR experiments in vaccinia. pAbT4537 is identical to pAbT4525, except that the tk flanking regions were

shortened and modified to contain additional, convenient restriction sites.

EXAMPLE 2: Recombination Vector Containing the 30K Vaccinia Promoter

This Example illustrates the construction of a monvalent in vivo recombination vector containing the vaccinia 30K promoter, with insertion at the vaccinia HindIII M region (Figure 4). The construction and structure of vectors pAbT3101 and pAbT3100 have been described in co-pending application Serial No. 910,501, incorporated herein by reference.

pAbT3101 was digested with EcoRI and SalI, and a 420bp fragment containing the vaccinia 30K promoter was gel-purified. pAbT3100, containing the vaccinia HindIII M fragment, was digested with SphI, treated with T4 DNA polymerase, ligated to EcoRI linkers (New England BioLabs), then digested with SalI, and a 3600bp fragment was gel-purified. The 3600bp and 420bp fragments were ligated to create pAbT3105, as shown in Figure 4A. pAbT3105 was digested with EcoRI and ligated to BamHI linkers (New England BioLabs) to create pAbT3106, as shown in Figure 4B. pAbT3106 was digested with BamHI and ligated to SacI linkers (New England BioLabs) to create pAbT3106S, as shown in Figure 4C.

pAbT4500 was digested with SphI and KpnI, and a 420bp fragment containing the vaccinia 30K promoter was gel-purified. pAbT4532B was digested with SphI and KpnI, and a 7300bp fragment was gel-purified and

ligated to the 420bp fragment to create pAbT4536, as shown in Figure 4D. pAbT4536 was digested with SalI and SacI, and a 420bp fragment containing the 30K promoter was gel-purified. pAbT3106S was digested with SalI and SacI, and a 3500bp fragment was gel-purified and ligated to the 420bp fragment to create pAbT4554, as shown in Figure 4E.

pAbT4554 is a plasmid vector for use in IVR experiments in vaccinia. pAbT4554 contains the vaccinia HindIII M region for directing recombination, including a portion of the 29K host-range gene for selection of recombinants, the 30K promoter followed by a multiple cloning site for the insertion and expression of foreign antigens, and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 3 Construction of a divalent in vivo recombination vector containing the vaccinia 30K and 40K promoters

pAbT4537 was digested with EcoRI and BamHI, and a 200bp fragment containing the vaccinia 40K promoter was gel-purified. pAbT4554 was partially digested with EcoRI and completely digested with BamHI and was ligated to the 200bp fragment to create pAbT4555, as shown in Figure 5A.

pAbT4555 is a divalent vector for use in IVR experiments in vaccinia. pAbT4555 contains the vaccinia HindIII M region for directing recombination, the vaccinia 29K host range gene for selection of recombinants, two vaccinia promoters, 30K and

40K, each followed by multiple cloning sites for the insertion and expression of foreign antigens, and a bacterial replicon and ampicillin-resistance gene for growth and selection of E. coli.

EXAMPLE 4 Trivalent in vivo recombination vector containing the vaccinia 30K, 40K and 7.5K promoters

pAbT4532B was digested with SmaI and XbaI, treated with Klenow and self-ligated to create pAbT4532X, as shown in Figure 5B. pAbT4532X was digested with HincII, ligated to SmaI linkers, digested with SmaI and SacI, and a 270bp fragment containing the vaccinia 7.5K promoter was gel-purified. pAbT4555 was digested with SmaI and SacI, and was ligated to the 270bp fragment to create pAbT4556, as shown in Figure 5C.

pAbT4556 is a trivalent vector for use in in vivo recombination experiments in vaccinia. pAbT4556 is identical to pAbT4555, except pAbT4556 contains three vaccinia promoters, 30K, 40K and 7.5K, each followed by multiple cloning sites for insertion and expression of foreign antigens.

EXAMPLE 5 Construction of a divalent in vivo recombinant vector containing the vaccinia 7.5K and 40K promoters

pAbT4027 (U.S. patent application, Serial No. 910,501) was digested with DraI and a 5530 bp fragment is gel-purified. pAbT4524B was partially digested with DraI. A 2260 bp fragment was gel-

purified and ligated to the 5530 bp fragment to create pAbT4533, as shown in Figure 6A.

pAbT4556 was digested with SphI and SmaI, and a 200bp fragment containing the vaccinia 40K promoter was gel-purified. pAbT4533 was digested with XbaI, treated with Klenow, and ligated to XhoI linkers (New England BioLabs; dCCTCGAGG) to create pAbT4533X, as shown in Figure 6B. pAbT4533X was digested with SphI and SmaI, and a 7400bp fragment was gel-purified and ligated to the 200bp fragment to create pAbT4586, as shown in Figure 6C.

pAbT4586 is a divalent vector for use in IVR experiments in vaccinia. pAbT4586 contains the vaccinia TK gene region for directing recombination, the BamF-lacZ gene for identification of vaccinia recombinants, the 7.5K and 40K promoters, each followed by a multiple cloning site for the insertion and expression of foreign antigens, and a bacterial replicon and ampicillin-resistance gene for growth and selection in E.coli.

EXAMPLE 6 Construction of a monovalent in vivo recombination vector containing the $SIV_{MAC-251}$ gene encoding envelope protein gp160 under the control of the vaccinia 40K promoter

Bacteriophage lambda EMBL4 containing full-length proviral SIV macaque strain 251 ($SIV_{MAC-251}$) DNA was obtained from Ronald C. Desrosiers (New England Regional Primate Research Center (NERPRC), Southborough, MA). This DNA, denoted lambda SIV_{251}, was digested with SacI and a 3500bp fragment

containing the gp160-encoding region (env) was gel-purified. pEMBL18 (Dente et al., (1983) Nucl. Acids Res. 11:1645) was digested with SacI and was ligated to the 3500bp fragment to create pAbT4566, as shown in Figure 7A.

To insert a convenient restriction site 5' to the initiation codon ATG of the $SIV_{MAC-251}$ env gene, two complementary oligonucleotides, DT210 and DT211, were synthesized (Dept. of Biology, Brandeis University); their sequences are shown in Figure 7B. pAbT4566 was partially digested with PvuII, and a 6500bp fragment containing all but the 5' 21bp of env and the 5' flanking region of env was gel-purified and ligated to DT210 and DT211 to create pAbT4096, as shown in Figure 7B. The env gene in pAbT4096 now has an XbaI site and an NcoI site 7 and 1bp, respectively, 5' to the env initiation codon ATG.

pAbT4096 was digested with XbaI and SacI, and a 2600bp fragment was gel-purified. pAbT4537 was partially digested with SacI and was completely digested with XbaI. The resulting 7300bp fragment was gel-purified and ligated to the 2600bp fragment to create pAbT4572, as shown in Figure 7C.

pAbT4572 is a vector for the insertion and expression of $SIV_{MAC-251}$ env in vaccinia. pAbT4572 contains the env gene under the control of the vaccinia 40K promoter, the DNA regions flanking the vaccinia TK gene for directing recombination in vaccinia, the lacZ gene under the control of the vaccinia BamF promoter for selection of vaccinia

recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E.coli (Figure 7C).

EXAMPLE 7 Construction of a monovalent in vivo recombination vector containing the $SIV_{MAC-251}$ gene encoding the gag polyprotein under the control of the vaccinia 40K promoter

Plasmid pHS251, containing the central portion of the $SIV_{MAC-251}$ proviral genome, was obtained from Ronald C. Desrosiers (NERPRC). This DNA was digested with KpnI, treated with T4 DNA polymerase, then was digested with ScaI, and a 2200bp fragment containing the gag gene was purified. This fragment also contains 900bp of the gene encoding the pol polyprotein, including the entire coding sequence of the protease required for the processing of the gag polyprotein; this fragment is therefore denoted as containing the gag-prot gene. pAbT4537 was digested with SmaI and was ligated to the 2200bp fragment to create pAbT4575 as shown in Figure 8A.

pAbT4575 is a vector for the insertion and expression of $SIV_{MAC-251}$ gag and protease in vaccinia. pAbT4575 is identical to pAbT4572 described in Example 6, except that pAbT4575 contains the gag-prot gene under the control of the vaccinia 40K promoter (Figure 8A).

Example 8 Construction of a divalent in vivo recombination vector containing the $SIV_{MAC-251}$ env gene under the control of the vaccinia 30K promoter and the $SIV_{MAC-251}$ gag-prot gene under the control of the vaccinia 40K promoter

pAbT4572 was digested with XbaI, treated with Klenow, then digested with SacI, and a 2600bp fragment containing the env gene was gel-purified. pAbT4554 was digested with SmaI and SacI, and was ligated to the 2600bp fragment to create pAbT4574, as shown in Figure 8B.

pAbT4574 was partially digested with EcoRI and was completely digested with Sa1I, and a 3000bp fragment containing the 30K promoter and env gene was gel-purified. pAbT4575 was digested with EcoRI and SacI, and a 2400bp fragment containing the 40K promoter and the gag-prot gene was gel-purified. pAbT4555 was digested with Sa1I and SacI, and a 3500bp fragment was gel-purified and ligated to the 3000bp and 2400bp fragments to create pAbT4577, as shown in Figure 8C.

pAbT4577 is a vector for the insertion and expression of $SIV_{MAC-251}$ env and gag-prot in vaccinia. pAbT4577 contains the env gene under the control of the vaccinia 30K promoter and the gag-prot gene under the control of the vaccinia 40K promoter, flanked by vaccinia DNA for directing recombination into the vaccinia HindIII M region. The vector DNA includes the 29K host-range gene for selection of vaccinia recombinants and a bacterial

replicon and ampicillin-resistance gene for growth and selection in E.coli (Figure 8C).

EXAMPLE 9 Construction of a monovalent in vivo recombination vector containing the $SIV_{MAC-251}$ 3'ORF gene under the control of the vaccinia 30K promoter

Plasmid p251PR3'ORF-I, containing a portion of of the $SIV_{MAC-251}$ proviral genome including the gene encoding 3'ORF, was obtained from Ronald C. Desrosiers (NERPRC). p251PR3'ORF-1 was digested with PstI and StuI, and an 800bp fragment containing the 3'ORF gene was gel-purified. pAbT4555 was digested with PstI and SmaI, and a 3900bp fragment was gel-purified and ligated to the 800bp fragment to create pAbT4576, as shown in Figure 9A.

pAbT4576 is a vector for the insertion and expression of $SIV_{MAC-251}$ 3'ORF in vaccinia. pAbT4576 contains the 3'ORF gene under the control of the vaccinia 30K promoter and contains the same vaccinia recombination and selection elements and bacterial components described for pAbT4577 in Example 8 (Figure 9A).

EXAMPLE 10 Construction of a monovalent in vivo recombination vector containing the $SIV_{MAC-251}$ sor gene under the control of the vaccinia 30K promoter

Lambda SIV_{251} DNA was digested with HindIII, and a 4300bp fragment containing a portion of the $SIV_{MAC-251}$ proviral genome including the pol and sor genes was gel-purified. pEMBL18 was digested with

HindIII and was ligated to the 4300bp fragment to create pAbT4579, as shown in Figure 10A.

pAbT4579 was digested with HincII and HphI, treated with T4 DNA polymerase, and a 1000bp fragment was gel-purified. pAbT4554 was digested with SmaI and was ligated to the 1000bp fragment to create pAbT4581, as shown in Figure 10B.

pAbT4581 is a vector for the insertion and expression of the $SIV_{MAC-251}$ sor gene in vaccinia. pAbT4581 is identical to pAbT4576 described in Example 9, except that pAbT4581 contains the sor gene under the control of the vaccinia 30K promoter (Figure 10B).

EXAMPLE 11 Construction of a monovalent in vivo recombination vector containing the $SIV_{MAC-251}$ pol gene under the control of the vaccinia 40K promoter

pAbT4579 was digested with HindIII and PstI, and a 1200bp fragment, containing the 5' end of the pol gene, was gel-purified. Bacteriophage m13mp18 DNA (New England BioLabs) was digested with HindIII and PstI, and was ligated to the 1200bp fragment to create pAbT4579A, as shown in Figure 11A.

The 5' end of the pol gene was modified to insert a convenient restriction site and a translation initiation ATG codon, by oligonucleotide-directed mutagenesis, as described in Materials and Methods. Using the oligonucleotide (Biology Dept., Brandeis University) shown in Figure 11B, a BamHI site and ATG codon were inserted at the beginning of

the pol-encoding region, creating pAbT4579B, as shown in Figure 11B. pAbT4579B was digested with BamHI and PstI, and a 900bp fragment containing the 5' end of pol was gel-purified. Bacteriophage m13mp19 DNA (New England BioLabs) was digested with BamHI and PstI, and was ligated to the 900bp fragment, to create pAbT4579C, as shown in Figure 11C.

pAbT4579 was digested with PstI, and a 2400bp fragment containing the 3' end of pol was gel-purified. pAbT4579C was digested with PstI, and was ligated to the 2400bp fragment, to create pAbT4582, as shown in Figure 11D. pAbT4582 was digested with BamHI and HindIII, and the 3300bp fragment containing the modified pol gene was gel-purified. pEMBL18 was digested with BamHI and HindIII, and was ligated to the 3300bp fragment to create pAbT4582B, as shown in Figure 11E. pAbT4582B was digested with HindIII, treated with Klenow, ligated to SacI linkers (New England BioLabs), digested with SacI, and a 3300bp fragment was gel-purified. pAbT4537 was partially digested with SacI and was ligated to the 3300bp fragment to create pAbT4583, as shown in Figure 11F.

pAbT4583 is a vector for the insertion and expression of $SIV_{MAC-251}$ pol in vaccinia. pAbT4583 is identical to pAbT4572 described in Example 6, except that pAbT4583 contains the pol gene under the control of the vaccinia 40K promoter (Figure 11F).

EXAMPLE 12 Construction of a divalent in vivo recombination vector

This vector contains the $SIV_{MAc-251}$ env gene under the control of the vaccinia 40K promoter and the $SIV_{MAC-251}$ gag-prot gene under the control of the vaccinia 7.5K promoter, with insertion at the vaccinia HindIII M region.

pAbT4575 was digested with BamHI and SacI, and a 1300bp fragment containing the 3' portion of the gag-prot gene was gel-purified. pHS251 was digested with KpnI and BamHI, and a 900bp fragment containing the 5' portion of the gag gene was gel-purified. pAbT4554 was digested with KpnI and SacI, and was ligated to the 1300bp and 900bp fragments to create pAbT4578, as shown in Figure 12A.

pAbT4574 was digested with BamHI, and a 2600bp fragment containing the env gene was gel-purified. pAbT4556 was digested with BamHI and was ligated to the 2600bp fragment to create pAbT4556A, as shown in Figure 12B.

pAbT4578 was digested with KpnI and SacI, and a 2600bp fragment containing the gag-prot gene was gel-purified. pAbT4556A was digested with KpnI and SacI, and was ligated to the 2600bp fragment to create pAbT4585, as shown in Figure 12C.

pAbT4585 is a vector for the insertion and expression of the $SIV_{MAC-251}$ env and gag-prot genes in vaccinia. pAbT4585 is identical to pAbT4577, except that pAbT4585 contains the env gene under the control of the 40K promoter and the gag-prot gene under the control of the 40K promoter and the gag-prot gene under the control of the 7.5K promoter (Figure 12C).

EXAMPLE 13 Construction of a divalent in vivo recombination vector

This vector contains the $SIV_{MAC-251}$ env gene under the control of the vaccinia 40K promoter and the $SIV_{MAC-251}$ gag-prot gene under the control of the vaccinia 7.5K promoter, with insertion at the vaccinia TK region.

pAbT4532B was partially digested with EcoRI and completely digested with HindIII, and a 3100bp fragment containg the lacZ gene was gel-purified. pAbT4585 was digested with EcoRI and SacI, and a 5600bp fragment containing the vaccinia 40K promoter, the $SIV_{MAC-251}$ env gene, the vaccinia 7.5K promoter and the $SIV_{MAC-251}$ gag-prot gene was gel-purified. pAbT4586 was digested with SacI and HindIII and a 4200bp fragment was gel-purified and ligated to the 3100bp and 5600bp fragments to create pAbT4589, as shown in Figure 13A.

pAbT4589 is a vector for the insertion and expression of $SIV_{MAC-251}$ env and gag-prot in vaccinia. pAbT4589 contains the SIV env gene under the control of the vaccinia 40K promoter, the SIV gag-prot gene under the control of the vaccinia 7.5K promoter, the DNA regions flanking the vaccinia TK gene for directing recombination in vaccina, the lacZ gene under the control of the vaccinia BamF promoter for selection of vaccinia recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E.coli (Figure 13A).

EXAMPLE 14 Construction of recombinant vaccinia viruses containing $SIV_{MAC-251}$ genes under the control of vaccinia promoters.

In vivo recombination is a method whereby recombinant vaccinia viruses are created (Nakano et al., (1982) Proc. Natl. Acad. Sci. USA 79:1593; Paoletti and Panicali, U.S. Patent No. 4,603,112). These recombinant viruses are formed by transfecting DNA containing a gene of interest into cells which have been infected by vaccinia virus. A small percent of the progeny virus will contain the gene of interest integrated into a specific site on the vaccinia genome. These recombinant viruses can express genes of foreign origin (Panicali and Paoletti. (1982) Proc. Natl. Acad. Sci. USA 79: 4927; Panicali et al., (1983) Proc. Natl. Acad. Sci. USA 80:5364).

$SIV_{MAC-251}$ genes were inserted into the vaccinia virus genome at either the HindIII J or HindIII M region, as listed in Table 2.

Table 2. SIV/Vaccinia Recombinants

Inserted at TK		Inserted at HindIII M	
Vaccinia Recombinant	Vaccinia Promoter $SIV_{MAC-251}$ Gene(s)	Vaccinia Recombinant	Vaccinia Promoter $SIV_{MAC-251}$ Gene(s)
vAbT193	40K env	vAbT198	30Kenv+40Kgag-prot
vAbT197	40K gag-prot	vAbT199	30K 3' orf
vAbT222	40K pol	vAbT206	30K sor
vAbT239	40K env+7.5Kgag-prot	vAbT223	40Kenv+7.5gag-prot

The TK gene is located in the HindIII J region. IVR vectors pAbT4572, 4575, 4583 or 4589 were transfected into Hu143TK$^-$ cells which had been infected with the NYCBH (TK$^+$) strain of vaccinia virus (see Materials and Methods). The selection system for recombinant virus was bromodeoxyuridine (BUdR), which is lethal for TK$^+$ virus but allows recombinant, TK$^-$ virus to grow. In addition, recombinant virus contains the lacZ gene which will metabolize Bluo-Gal and turn recombinant plaques blue. Therefore, blue, TK$^-$ plaques were picked and purified, and were shown, by Southern analysis, to contain the appropriate $SIV_{MAC-251}$ gene(s): vAbT193 contains env; vAbT197, gag-prot; vAbT222, pol; vAbT239, env + gag-prot.

The 29K host-range gene is located in the HindIII M region (Gillard et al., (1986) Proc. Natl. Acad. Sci. USA 83: 5573) and has been developed as a selection system. Recombinant vaccinia virus vAbT33 contains the lacZ gene in place of the 29K gene. Therefore vAbT33 cannot grow on RK13 cells which require the 29K gene product, and, on permissive cells, is blue in the presence of BluoGal. See U.S. Patent Application Serial No. 205,189, filed concurrently herewith.

IVR vectors pAbT4577, 4576, 4581 or 4585 were transfected into BSC-40 cells which had been infected with vaccinia virus vAbT33 (See Materials and Methods). Recombinant viruses were selected as white plaques in the presence of BluoGal on RK13 cells. Plaques were picked and purified, and were

shown, by Southern analysis, to contain the appropriate $SIV_{MAC-251}$ gene(s): vAbT198 contains env + gag-prot; vAbT199, 3' orf; vAbT206, sor; vAbT223, env + gag-prot.

EXAMPLE 15 Black plaque assay for expression of $SIV_{MAC-251}$ antigens in recombinant vaccinia.

The black plaque assay, described in Materials and Methods, is an in situ enzyme-based immunoassay which can detect protein expressed by vaccinia-infected cells.

The black plaque assay was performed on vaccinia recombinants vAbT193, 197, 198, 199, 206, 222 and 223, using SIV-infected macaque serum 127-87 obtained from Ronald C. Desrosiers (NERPRC).

Plaques formed by the negative control, NYCBH virus, showed only a background color which was consistent with the background on the cell monolayer itself. Plaques formed by vaccinia recombinants vAbT193, 197, 198, 223 and 239 stained a distinct dark purple color which was much darker than the background on the cell monolayer, showing that these recombinants strongly express $SIV_{MAC-251}$ antigen(s). Black plaque assays of vAbT199, 206 and 222 were weakly positive.

EXAMPLE 16 Immunoprecipitation of $SIV_{MAC-251}$ antigens from recombinant vaccinia

Immunoprecipitation analysis was performed on cells infected with recombinant vaccinia virus vAbT193, 197, 198, 222 and 223, as described in Materials and Methods. The results are summarized in Table 3.

Table 3. Immunoprecipitation of $SIV_{MAC-251}$ proteins from recombinant vaccinia

Vaccinia Recombinant	SIV gene(s)	Proteins observed
vAbT193	env	gp160, gp120, gp32
vAbT197	gag-prot	p55, p40, p24, p15
vAbT198	env, gag-prot	gp160,gp120,p55, p40,p24,p15
vAbT222	pol	p64, p53, p10
vAbT223	env, gag-prot	gp160,gp120,gp32, p55,p40,p24,p15

Each recombinant contains $SIV_{MAC-251}$ proteins of the predicted correct sizes: the env-containing recombinants contain the precursor gp160 and the processed gp120/gp32; the gag-prot-containing recombinants contain predominantly the precursor p55 but also the 40K intermediate and the processed p24 and p15; the pol-containing recombinants contain the p64, p53 and p10 processed products. Thus each of these vaccinia recombinants is expressing the appropriate

$SIV_{MAC-251}$ antigen and is also undergoing correct processing of these antigens.

EXAMPLE 17 Enzyme-linked immunosorbant assay (ELISA) with immune sera of mice immunized with recombinant vaccinia virus expressing $SIV_{MAC-251}$ antigens

Mice were immunized with vaccinia recombinants vAbT193, 197, 198 or 223, as described in Materials and Methods.

Sera were obtained three weeks post-immunization and tested against vaccinia or against SIV by ELISA, as described in Materials and Methods. The results are shown in Table 3. Most mice exhibited a good anti-vaccinia immune response against the vaccinia recombinants, and a good anti-SIV response was observed with vaccinia recombinants vAbT198 and 223.

EXAMPLE 18

SIV neutralization assay with immune sera of mice immunized with recombinant vaccinia virus expressing $SIV_{MAC-251}$ antigens.

The mouse sera described in Example 17 were tested in an SIV neutralization assay, as described in Materials and Methods.

Results are shown in Table 4. Antisera from all the vAbT198-immunized mice and one vAbT223-

immunized mouse efficiently neutralized SIV; anti-sera from the vAbT197-immunized mice exhibited lower neutralization activity; the other antisera did not neutralize SIV.

Table 4. Characterization of Mouse Immune Sera

Vaccinia Recombinant	Mouse Serum	Anti-vaccinia antibody titer	Anti-SIV antibody titer	SIV neutralizing antibody titer
vAbT193: 40Kenv	C3	1:100	1:20	1:20
	C4	1:200	1:20	1:80
	C5	1:100	<1:20	1:20
vAbT197: 40Kgag	D1	1:3200	1:20	<1:10
	D2	1:1600	<1:20	<1:10
	D3	1:1600	1:20	<1:10
	D4	1:1600	<1:20	<1:10
	D5	1:800	<1:20	<1:10
vAbT198: 30K env 40K gag	E1	1:12,800	1:1280	1:640
	E2	1:6400	1:1280	1:640
	E3	1:12,800	1:1280	1:640
	E4	1:6400	1:1280	1:640
vAbT223: 40K env 7.5K gag	C1	1:1600	1:1280	1:20
	C2	1:50	<1:20	1:20
	C3	1:6400	1:2560	1:1280
	C4	<1:50	1:20	<1:20
-	Normal Balb/c			<1:20

Deposit

The recombinant plasmid pAbT 4585 has been placed on deposit at the American Type Culture Collection in Rockville, Maryland, U.S.A. The plasmid has been assigned the accession number 67729.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A recombinant pox virus capable of expressing in a host animal one or more proteins of simian or human immunodeficiency virus.

2. A recombinant pox virus of claim 1, which is of the species vaccinia.

3. A recombinant pox virus of claim 1, wherein the proteins are selected from the group consisting of polypeptides encoded by the env, gag, pol, sor, 3'-orf, art, and tat genes of SIV as shown in Figure 1 or the HIV counterparts thereof.

4. A recombinant pox virus of claim 3, wherein the expressed proteins consist of a fragment or portion of the complete protein sequence encoded by the SIV or HIV gene.

5. A recombinant vaccinia virus containing, in a region of the viral genome nonessential for replication of the virus, one or more genes of simian immunodeficiency virus, the gene or genes being under control of individual vaccinia promoters.

6. A recombinant vaccinia virus of claim 5, wherein the SIV genes are selected from the group consisting of the env, gag, pol, sor,

3'-orf, tat, and art genes, or fragments thereof.

7. A vector for insertion of SIV or HIV DNA sequences and for subsequent in vivo recombination with pox virus to produce a recombinant pox virus capable of expressing the SIV or HIV DNA in a host, comprising;

a. a pox virus promoter linked to;

b. a DNA sequence encoding an SIV or HIV antigen; the DNA sequence being under control of the promoter; and;

c. DNA sequences of the pox virus flanking the construct of elements a and b, the DNA sequences being substantially homologous to a region of the pox viral genome which is nonessential for replication of the pox virus;

d. a replicon for replication in a prokaryotic host; and

e. a structural gene encoding a marker or indicator for selection of prokaryotic hosts transformed with the vector, the gene being under control of a prokaryotic promoter.

8. A vector of Claim 7 for recombination with vaccinia virus, wherein the pox virus promoter is the 11K promoter, the 7.5K promoter, the 40K promoter, the BamF promoter or the 30K promoter of vaccinia virus and where the flanking DNA

sequences are sequences homologous to the thymidine kinase gene of vaccinia virus.

9. A vector of Claim 7, further comprising:

f. a pox virus promoter linked to a structural gene which encodes a marker or indicator for selection of recombinant pox virus, the promoter and structural gene being located between the flanking pox viral sequences.

10. A vector of Claim 9, for recombination with vaccinia virus, wherein the pox virus promoter is the vaccinia BamF promoter and the structural gene encoding a marker or indicator is the E. coli lacZ gene encoding B-galactosidase.

11. A method of evaluating the effectiveness of a recombinant pox virus expressing antigens of simian immunodeficiency virus to protect non-human primates against acquisition of SIV and development of simian AIDS, comprising the steps of:

a. inoculating a monkey with a recombinant pox virus capable of expressing one or more SIV polypeptides;

b. allowing the recombinant pox virus to replicate within the non-human primate host;

c. challenging inoculated monkey with pathogenic SIV; and

d. determining whether or nor the monkey acquires the virus and develops simian AIDS.

12. A method of Claim 11 wherein the pox virus is the vaccinia virus.

13. A means of Claim 11, wherein the monkey is the macaque monkey.

14. A method of evaluating the effectiveness of a recombinant pox virus expressing HIV polypeptides to protect humans against acquisition of HIV and development of human AIDS, comprising the steps of:

a. inoculating a non-human primate with a pox virus capable of expressing one or more immunogenic SIV polypeptides, the SIV polypeptides being counterparts of the polypeptides found in the HIV genome;

b. allowing the recombinant pox virus to replicate within the non-human primate host;

c. challenging inoculated and uninoculated hosts with pathogenic SIV; and

d. determining whether or not the non-human primate acquires the virus and develops simian AIDS as indicative of the effectiveness of the recombinant.

15. A method of Claim 14 wherein the non-human primate is the macaque monkey and the pox virus is the vaccinia virus.

16. A method of Claim 14, wherein the SIV polypeptides that have their counterpart in the HIV genome are selected from the group consisting of the gag, env, pol, sor, 3'orf, art and tat sequences.

17. A vector for insertion of SIV or HIV DNA sequences and for subsequent in vivo recombination with pox virus to produce a recombinant pox virus capable of expressing the polypeptides encoded by the SIV or HIV DNA in a host, comprising:

a. one or more pox virus promoters linked to:

b. DNA sequences encoding HIV or SIV antigens, each DNA sequence being under the control of a different pox virus promoter;

c. DNA sequences of the pox virus flanking the construct of elements a and b, the DNA sequences being substantially homologous to a region of the pox viral genome which is nonessential for replication of the pox virus;

d. a replicon for replication in a prokaryotic host; and

e. a structural gene encoding a marker or indicator for selection of prokarytoic hosts transformed by the vector, the gene

being under control of a prokarytoic promoter.

18. A vector of Claim 17, wherein the pox virus promoter is selected from the group consisting of the 7.5K promoter, the 40K promoter, the BamF promoter, the 11K promoter and the 30K promoter.

19. A vector of Claim 18, for recombination with a pox virus that contains a mutation in the 29K gene, wherein the flanking DNA sequences are:
a. homologous to the Hind IIIM region of vaccinia virus; and
b. capable of restoring the function of the 29K gene in the recombinant viruses.

20. A vector of Claim 17, for recombination with vaccinia virus, wherein the pox virus promoter is the vaccinia 30K promoter and the SIV DNA sequences are those encoding the sor gene product.

21. A vector of Claim 17, for recombination with vaccinia virus, wherein the pox virus promoter is the vaccinia 30K promoter, and the SIV DNA sequences are those encoding the 3'-orf gene product.

22. A vector of Claim 18, for recombination with vaccinia virus, wherein the pox virus promoters

are the vaccinia 30K and 40K promoters, and the SIV DNA sequences are those encoding:

a. the SIV env gene products under the control of the 30K promoters; and

b. the SIV gag-prot gene products, under the control of the 40K promoter.

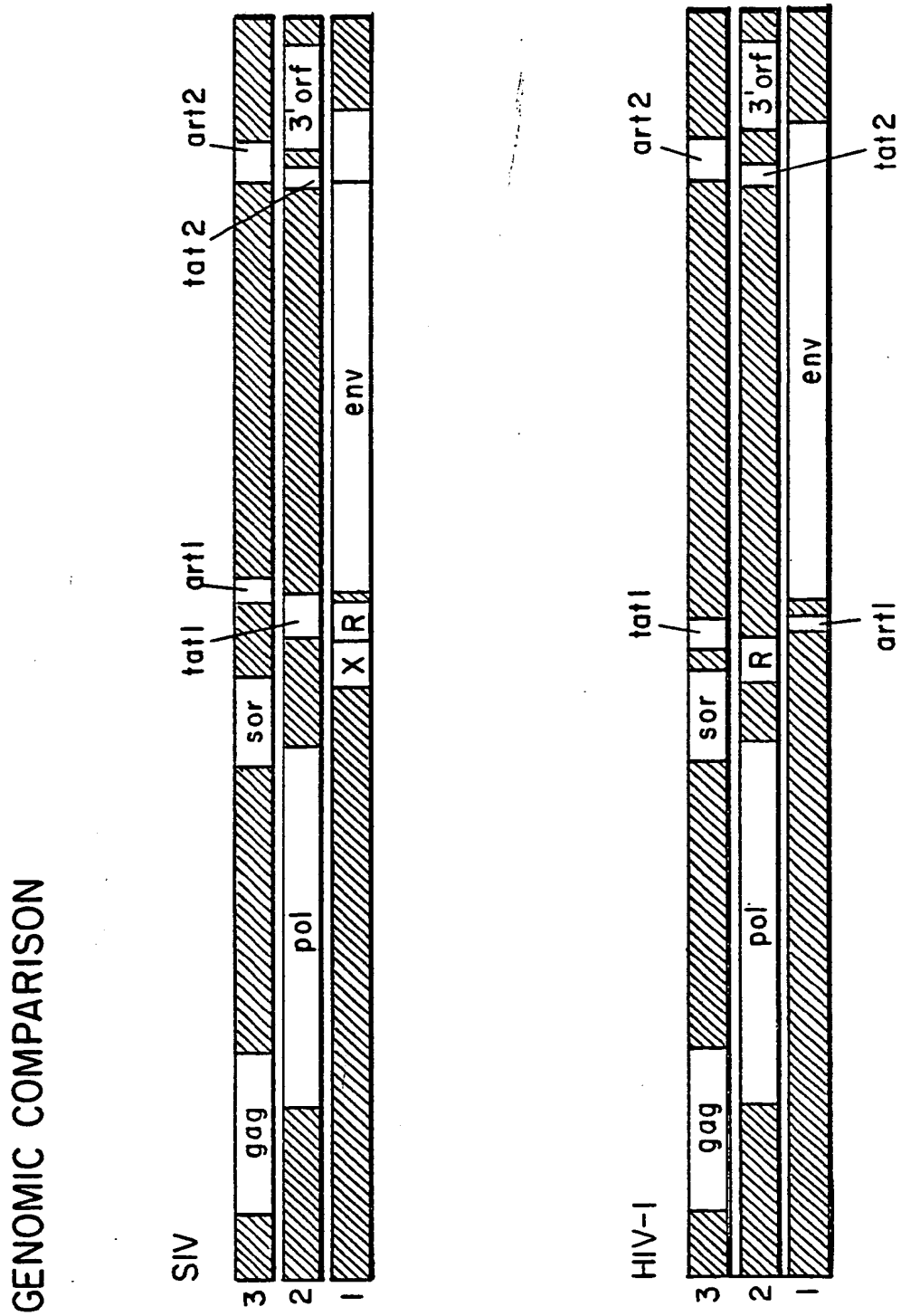
23. A vector of Claim 18, for recombination with vaccinia virus, wherein the pox virus promoters are the vaccinia 40K and 7.5K promoters, and the SIV DNA sequences are those encoding:

a. the SIV env gene products under the control of the 40K promoter; and

b. the SIV gag-prot gene products, under the control of the 7.5K promoter.

24. Recombinant vaccinia viruses having the characteristics of vAbT193, vAbT197, vAbT198, vAbT199, vAbT206, vAbT222, vAbT223, vAbT239.

FIG. 1



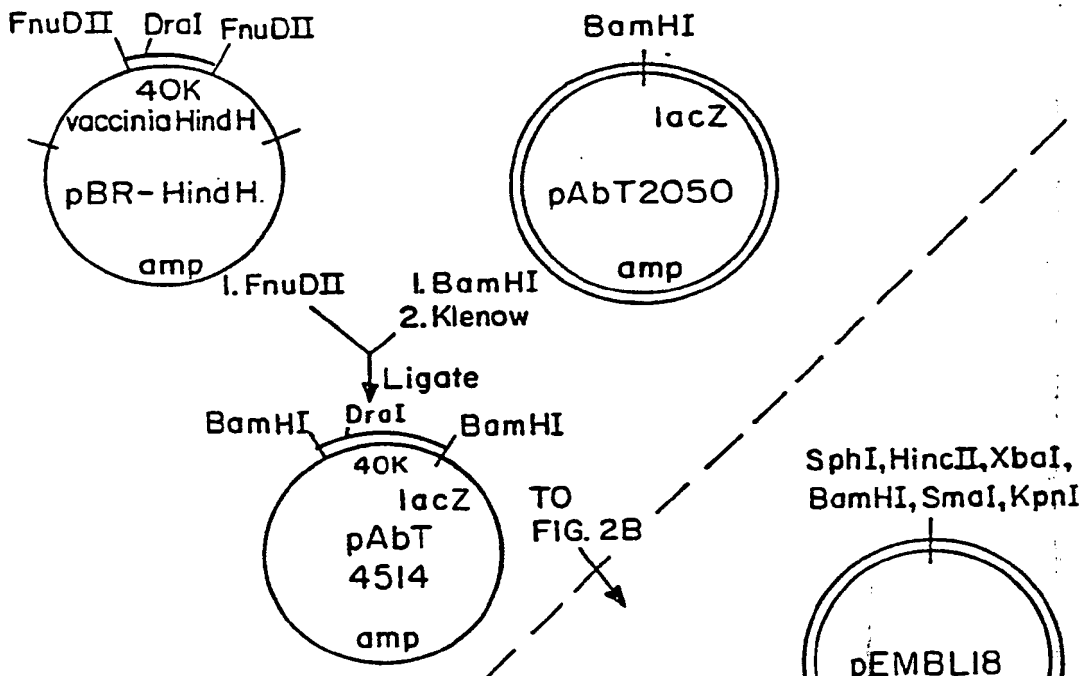


FIG. 2A

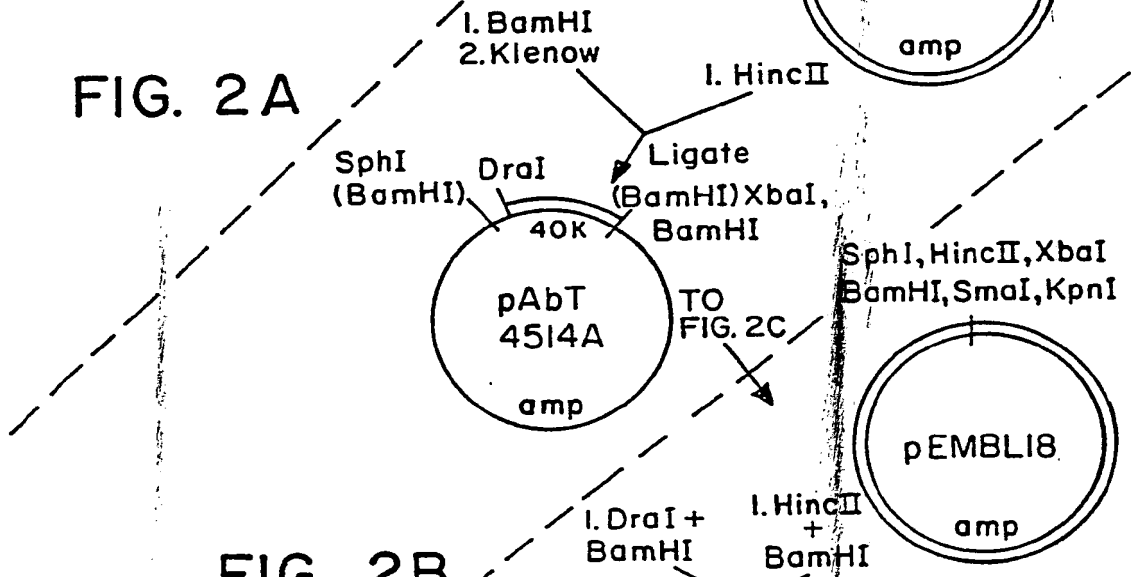


FIG. 2B

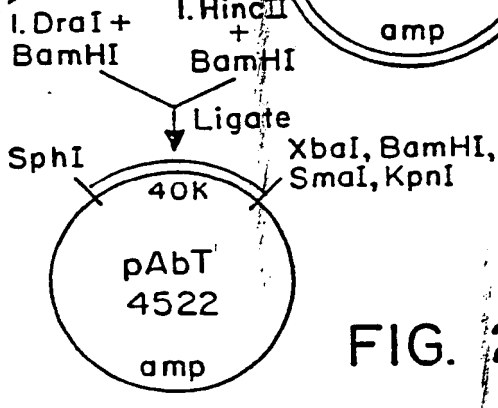


FIG. 2C

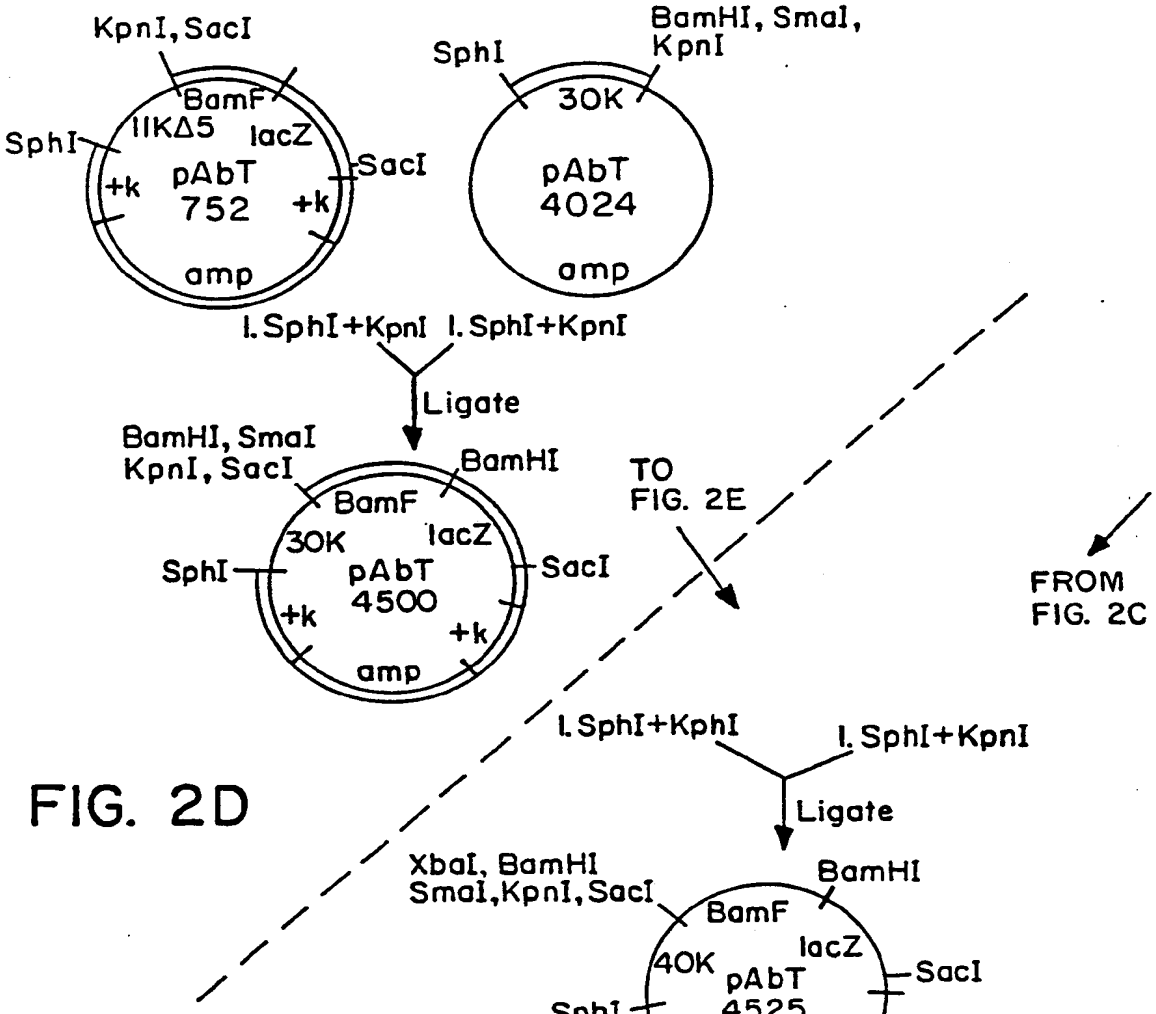


FIG. 2D

FIG. 2E

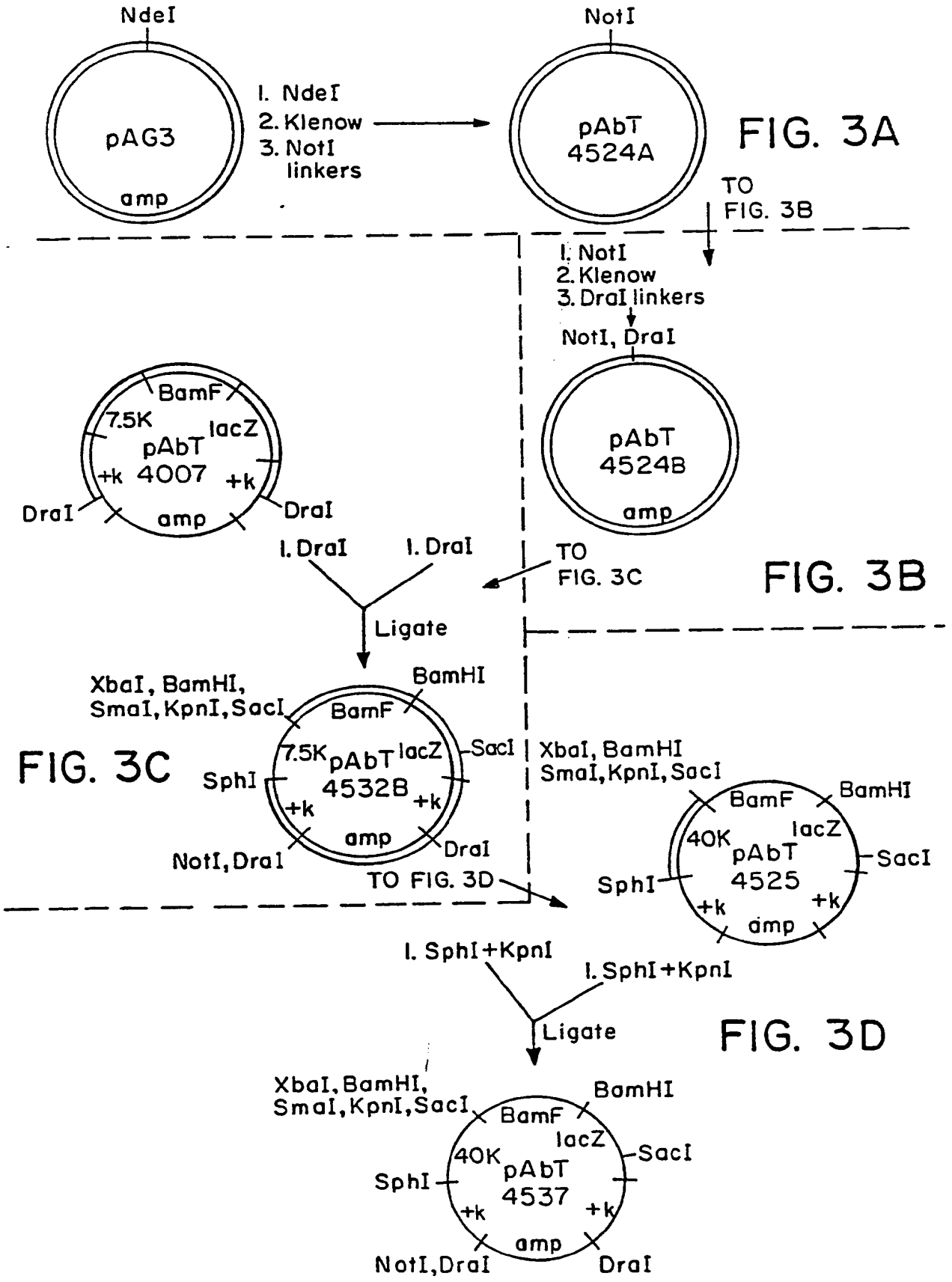


FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

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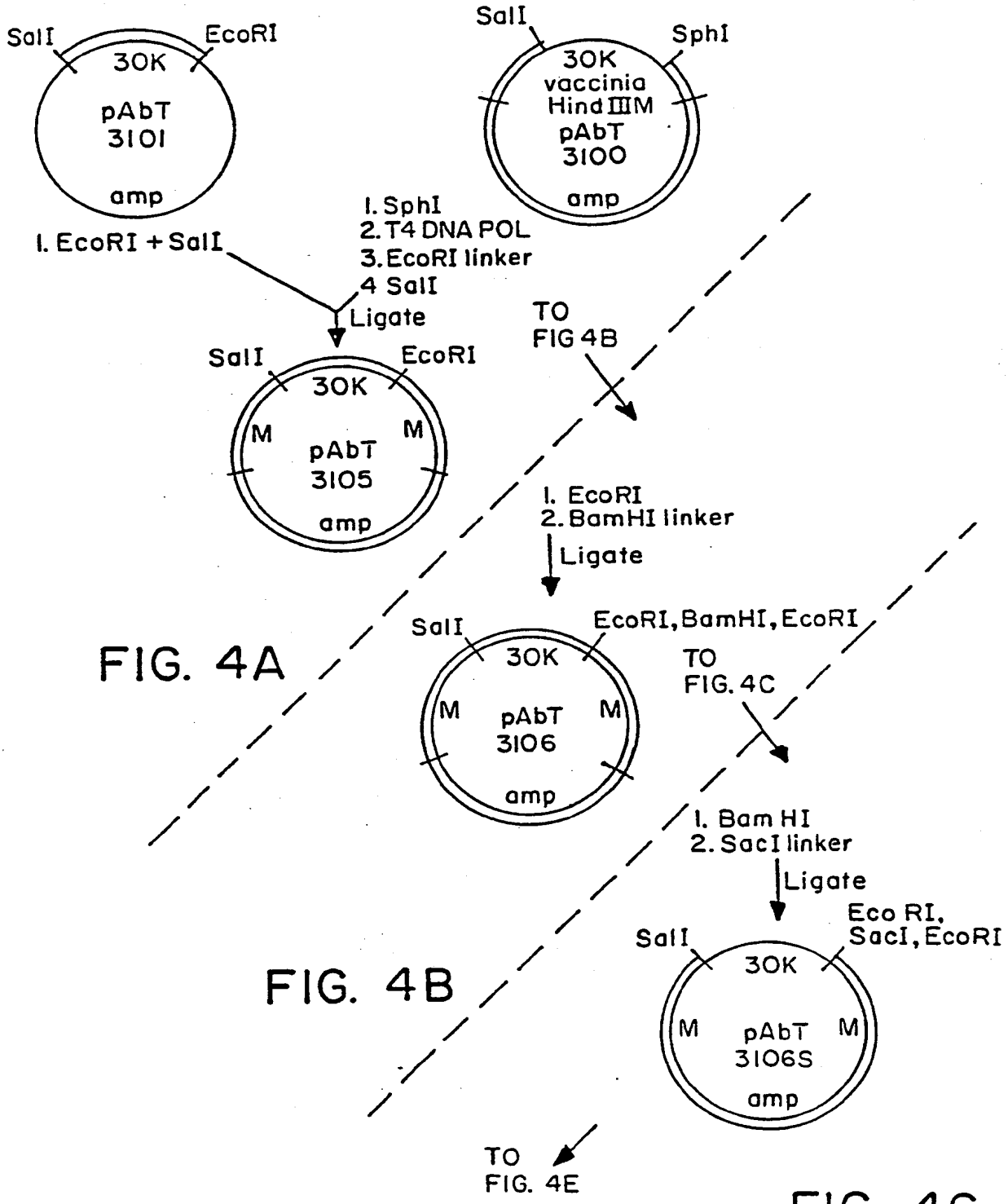

FIG. 4A

FIG. 4B

FIG. 4C

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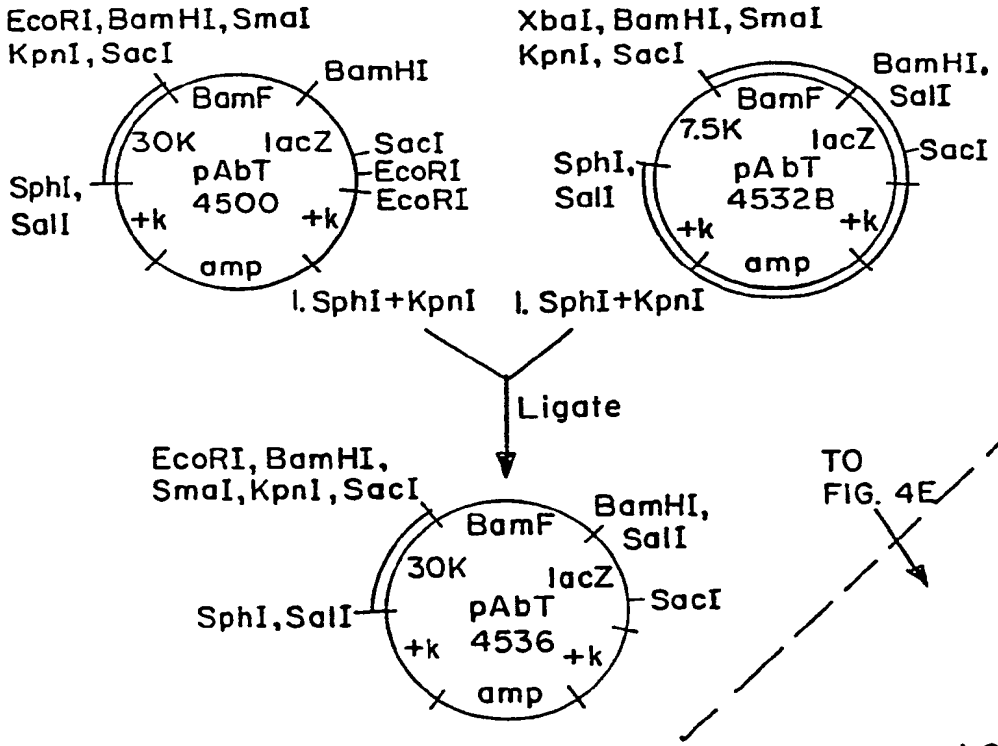


FIG. 4D

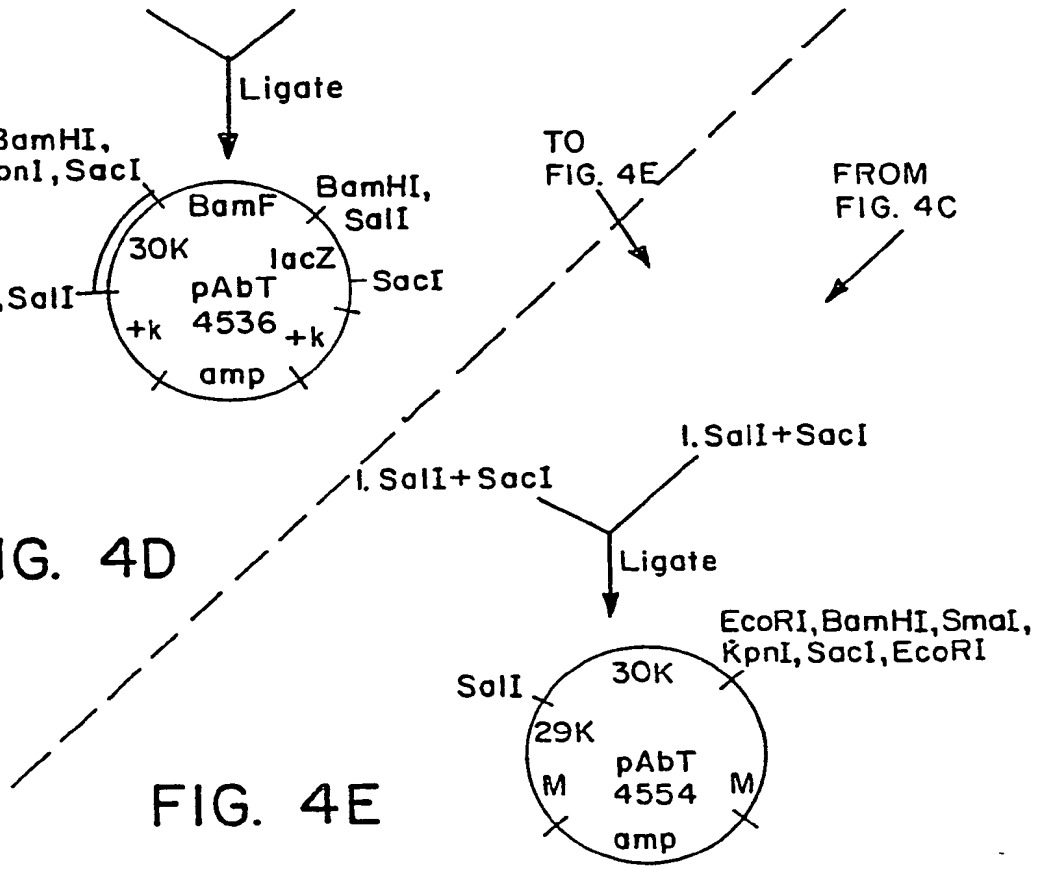
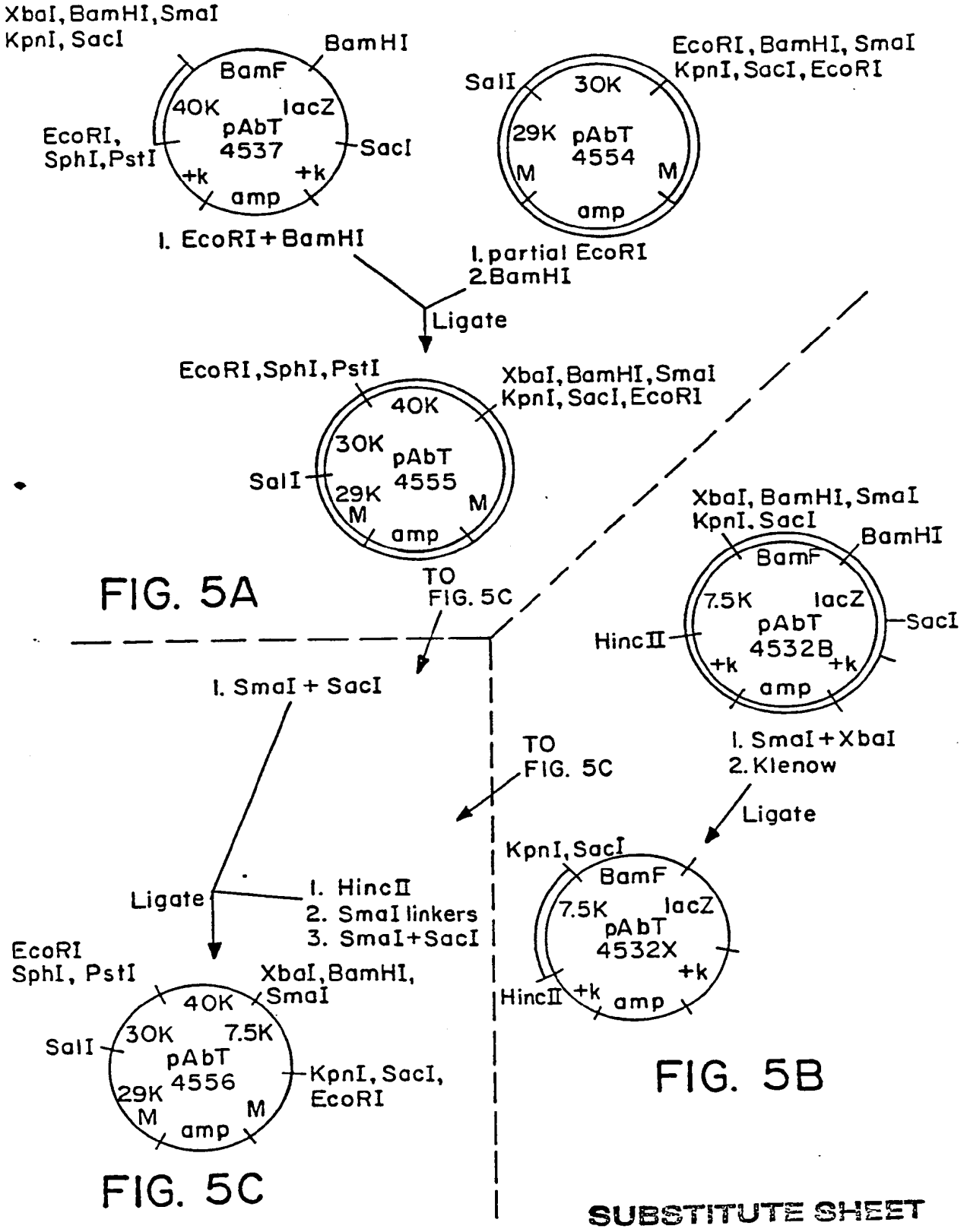


FIG. 4E



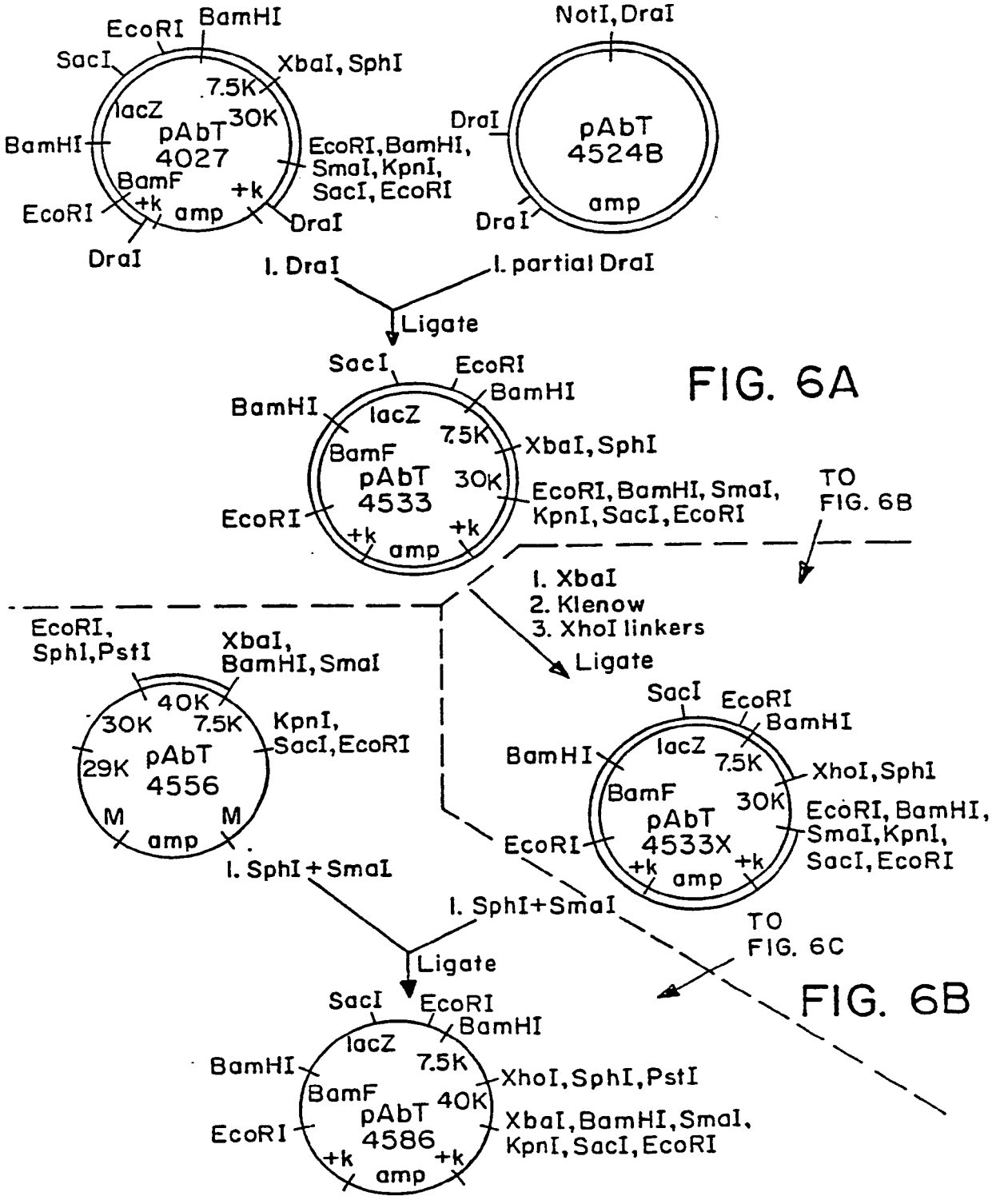


FIG. 6A

FIG. 6B

FIG. 6C

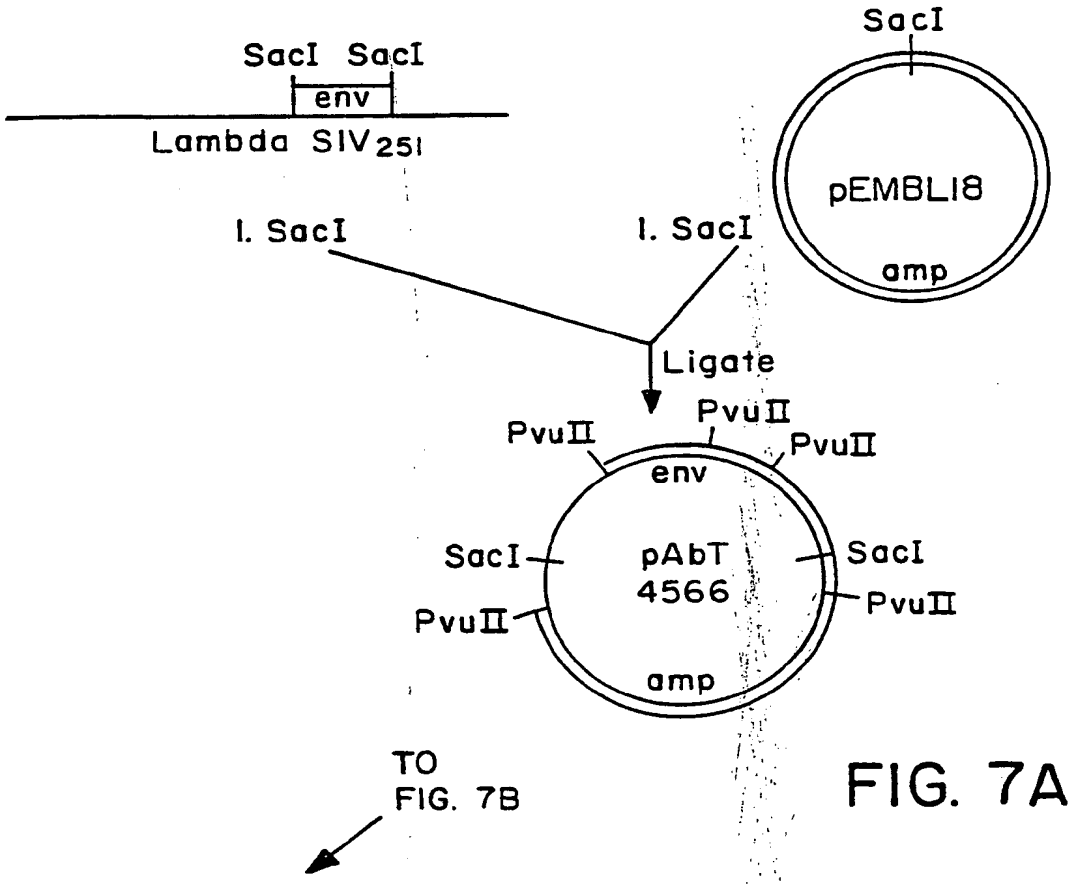
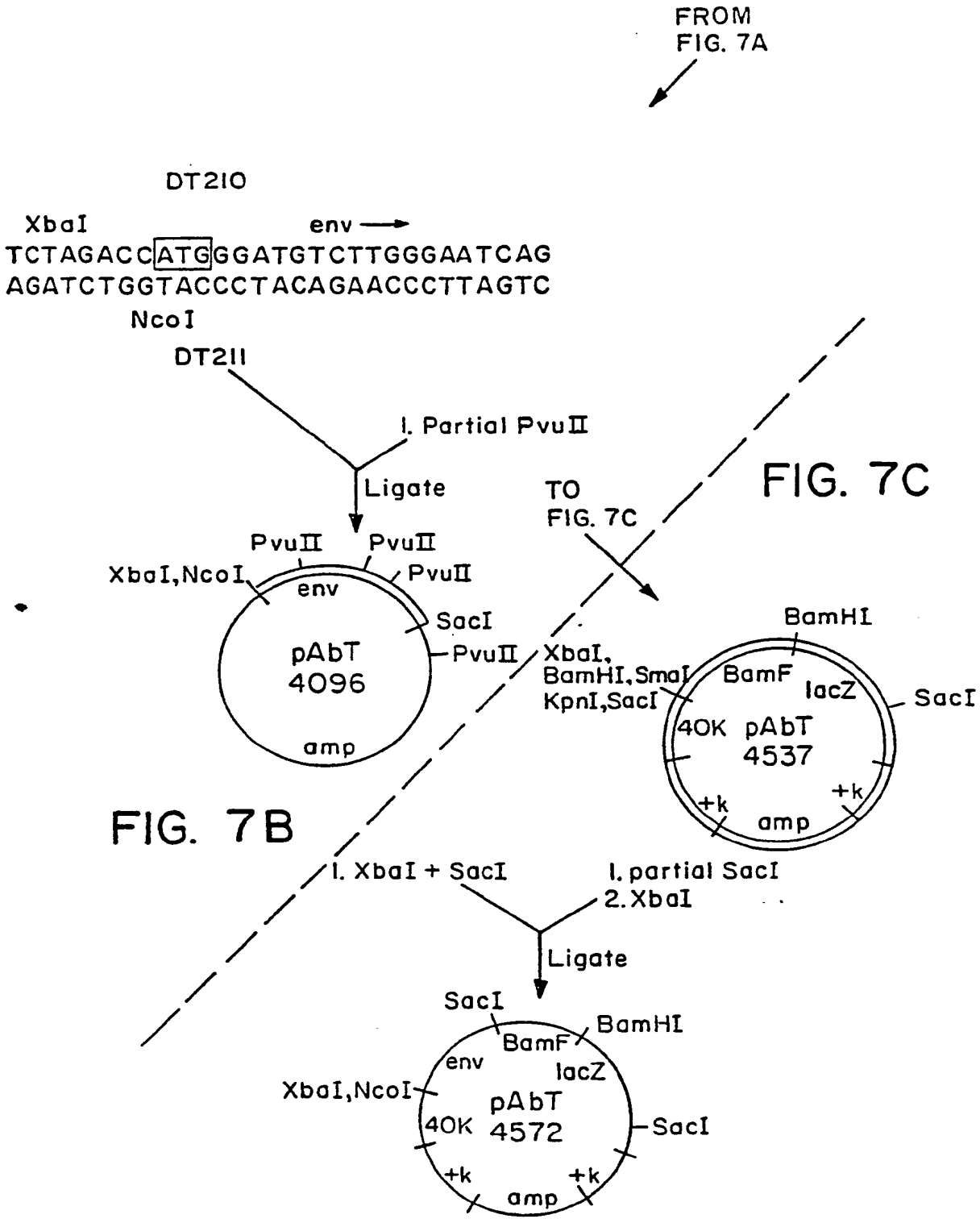
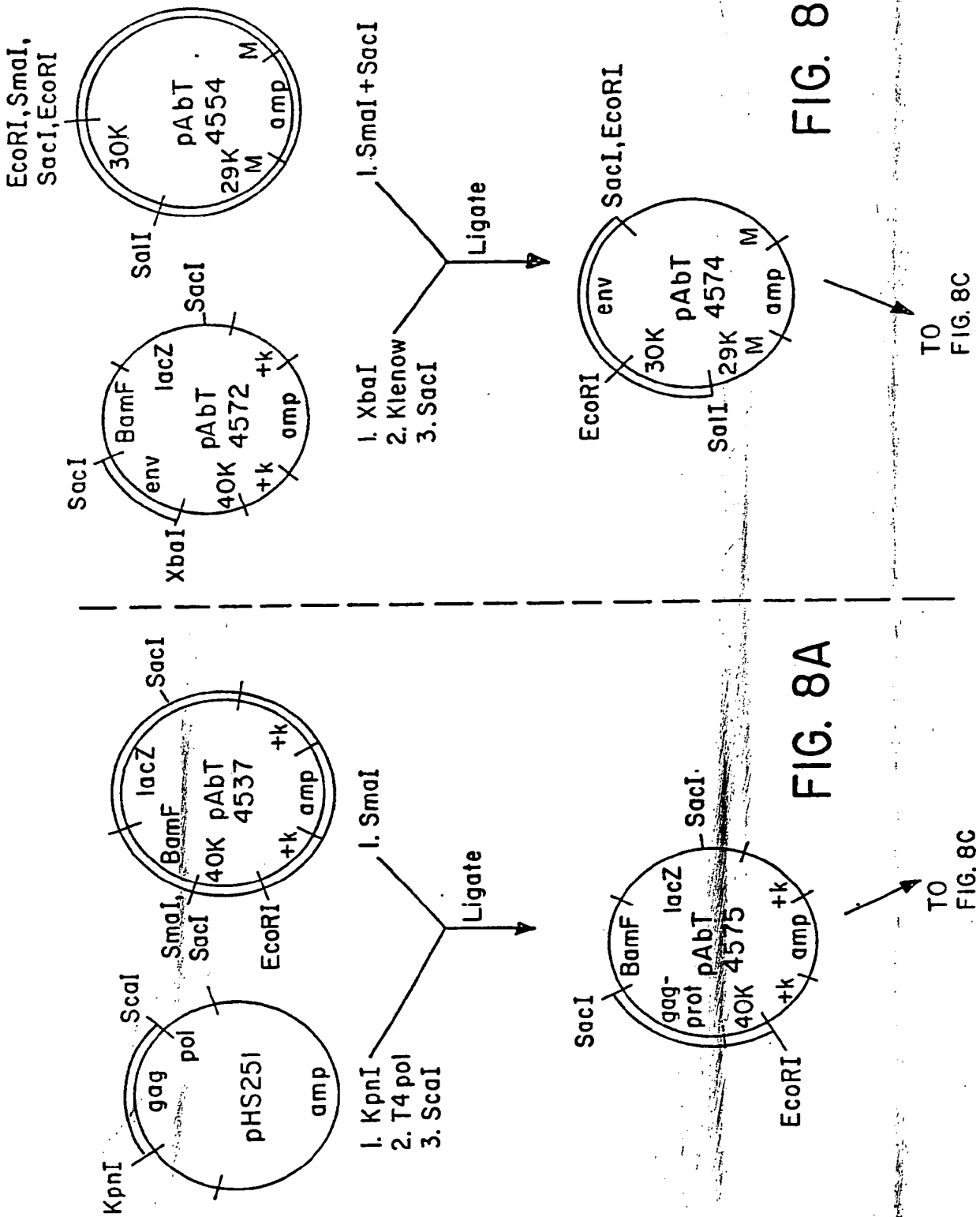


FIG. 7A





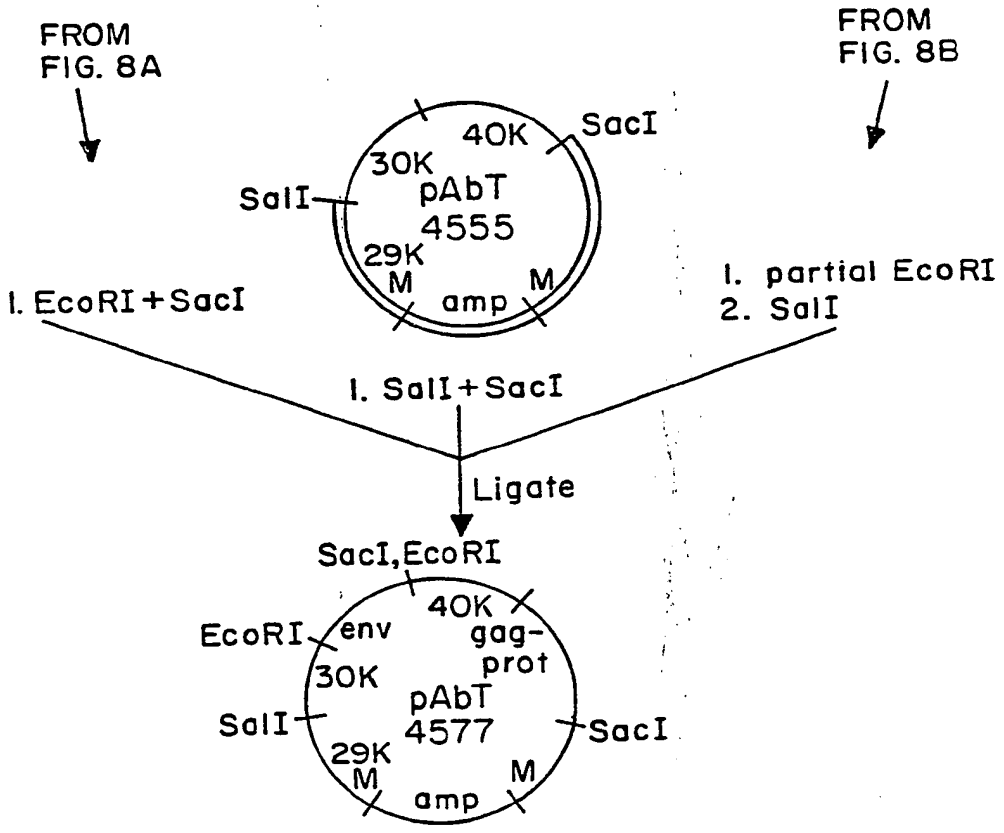


FIG. 8C

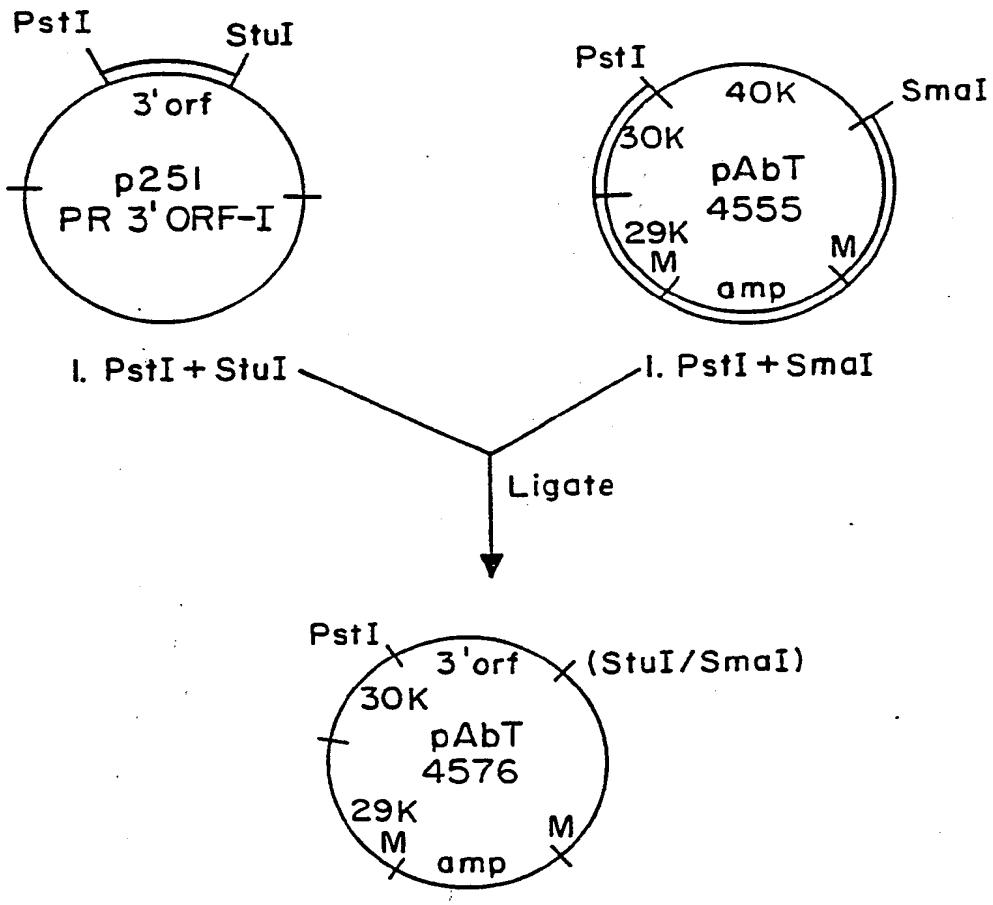


FIG. 9

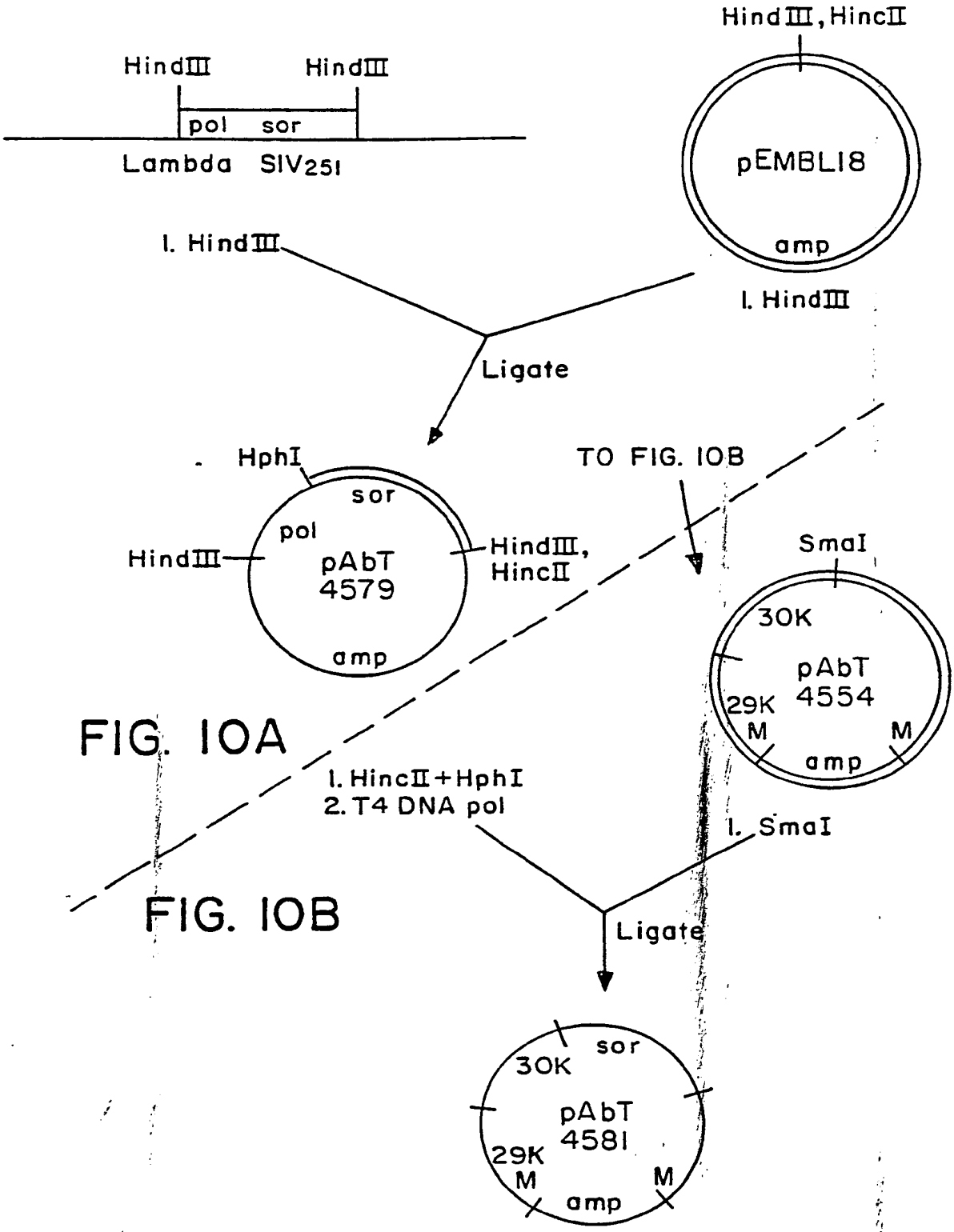
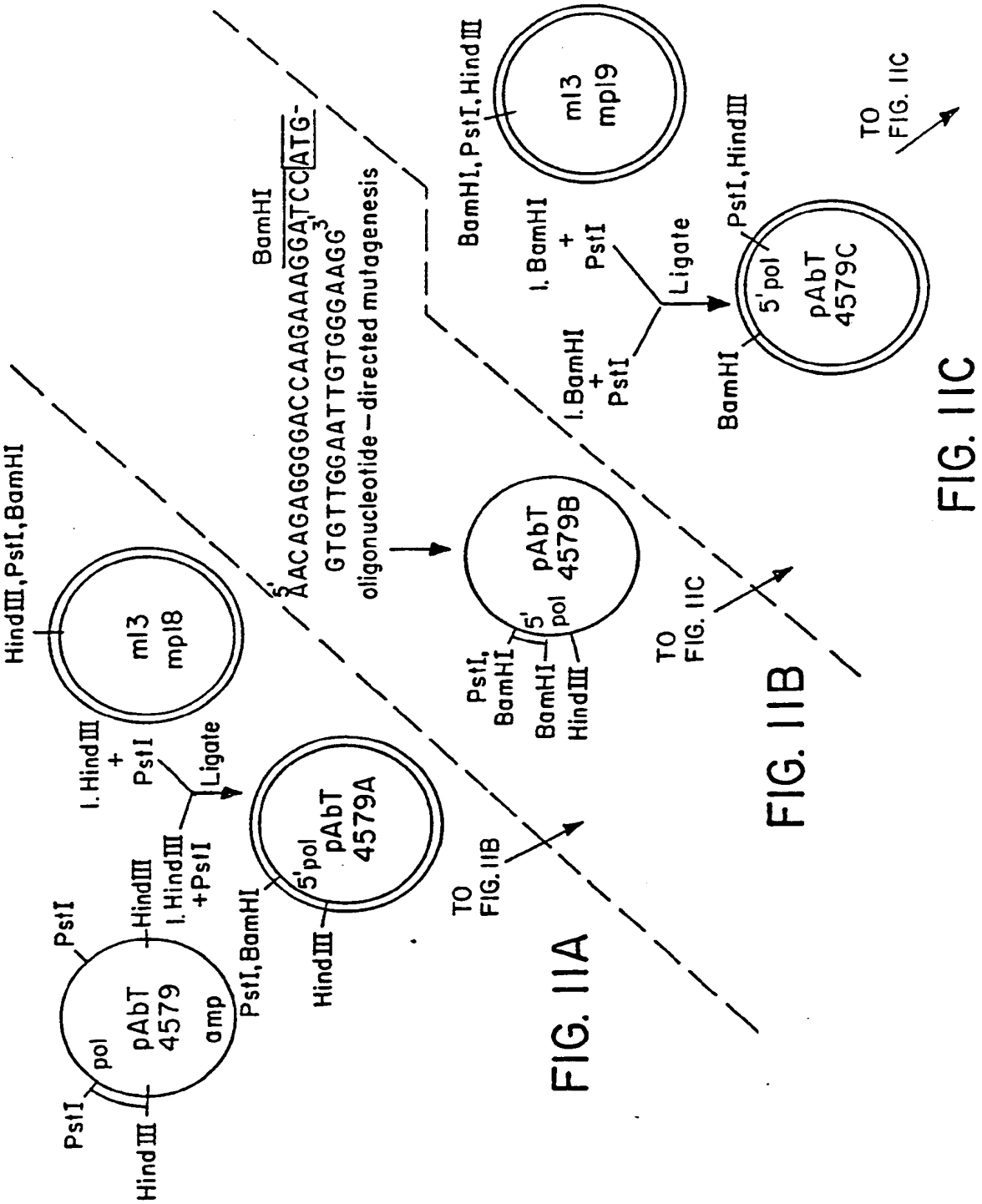
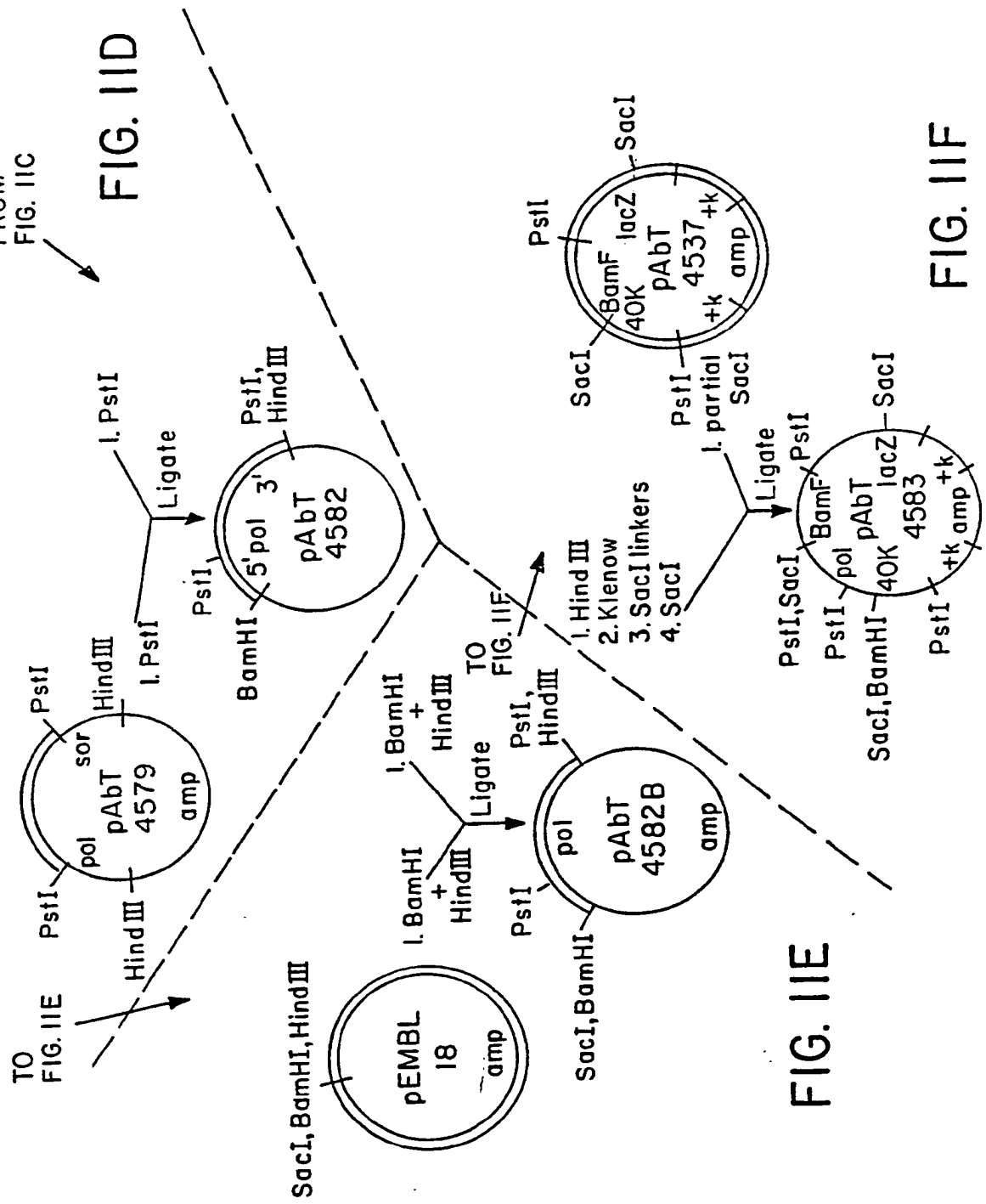


FIG. 10A

FIG. 10B





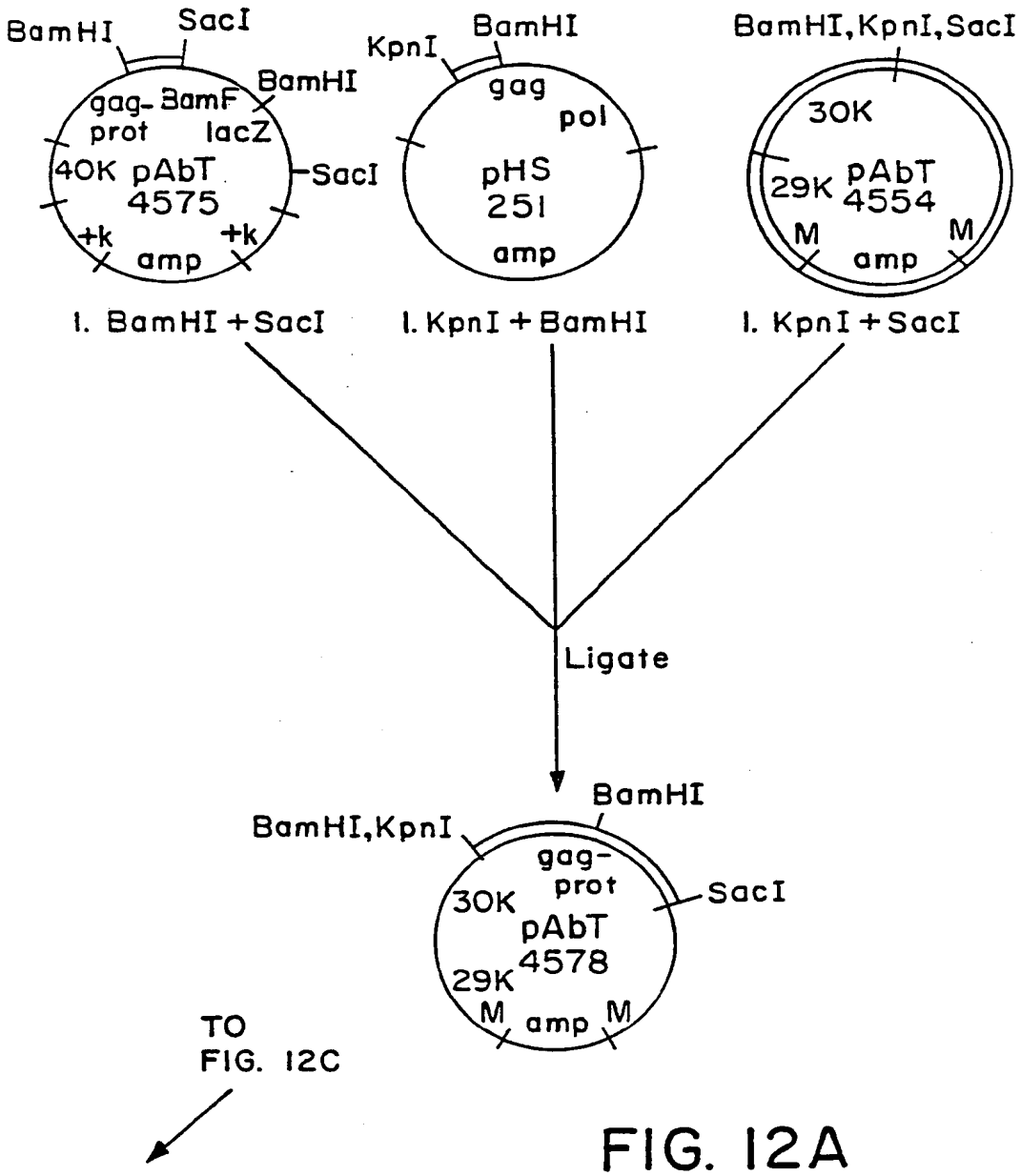


FIG. 12A

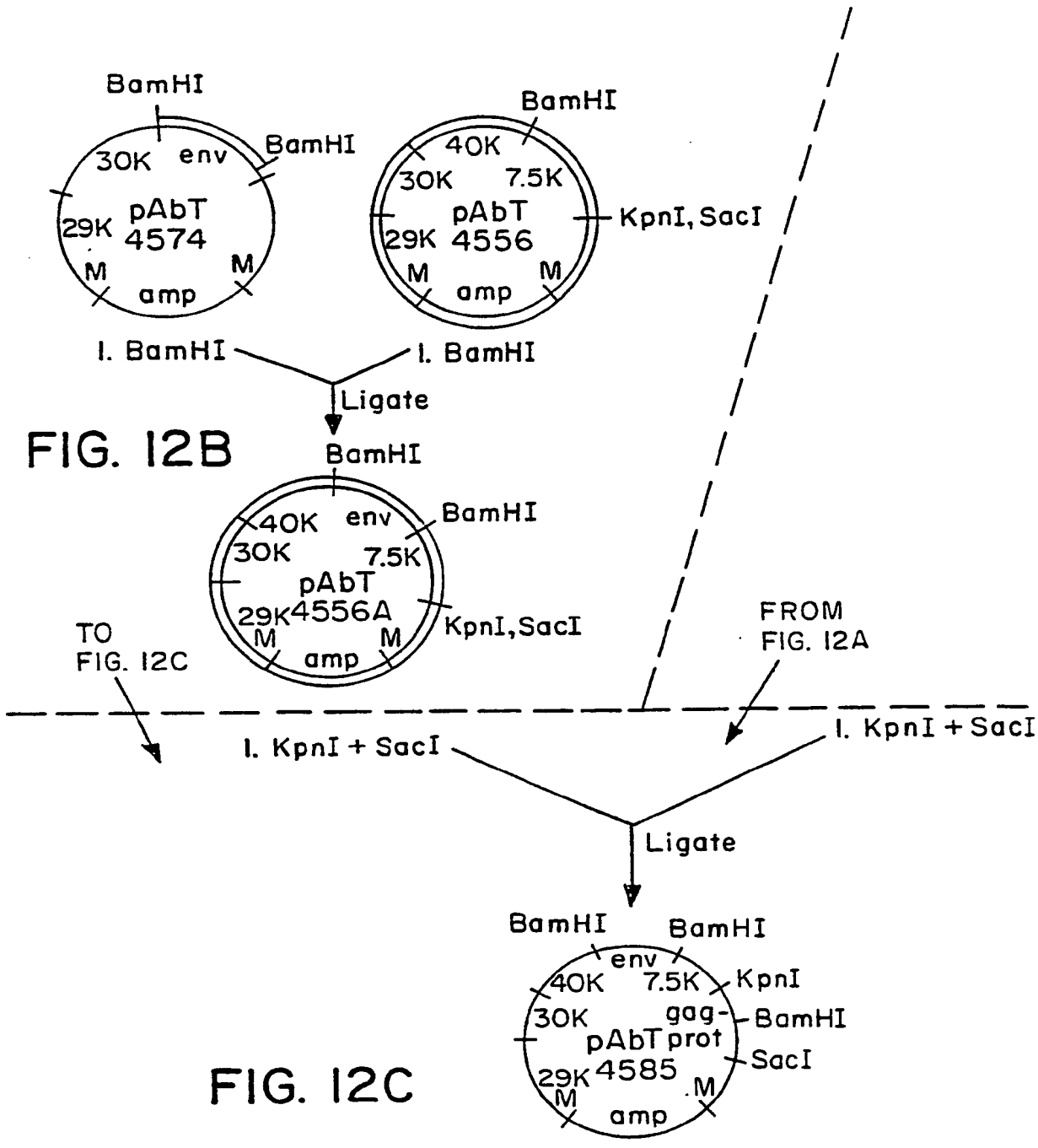


FIG. 12B

FIG. 12C

SUBSTITUTE SHEET

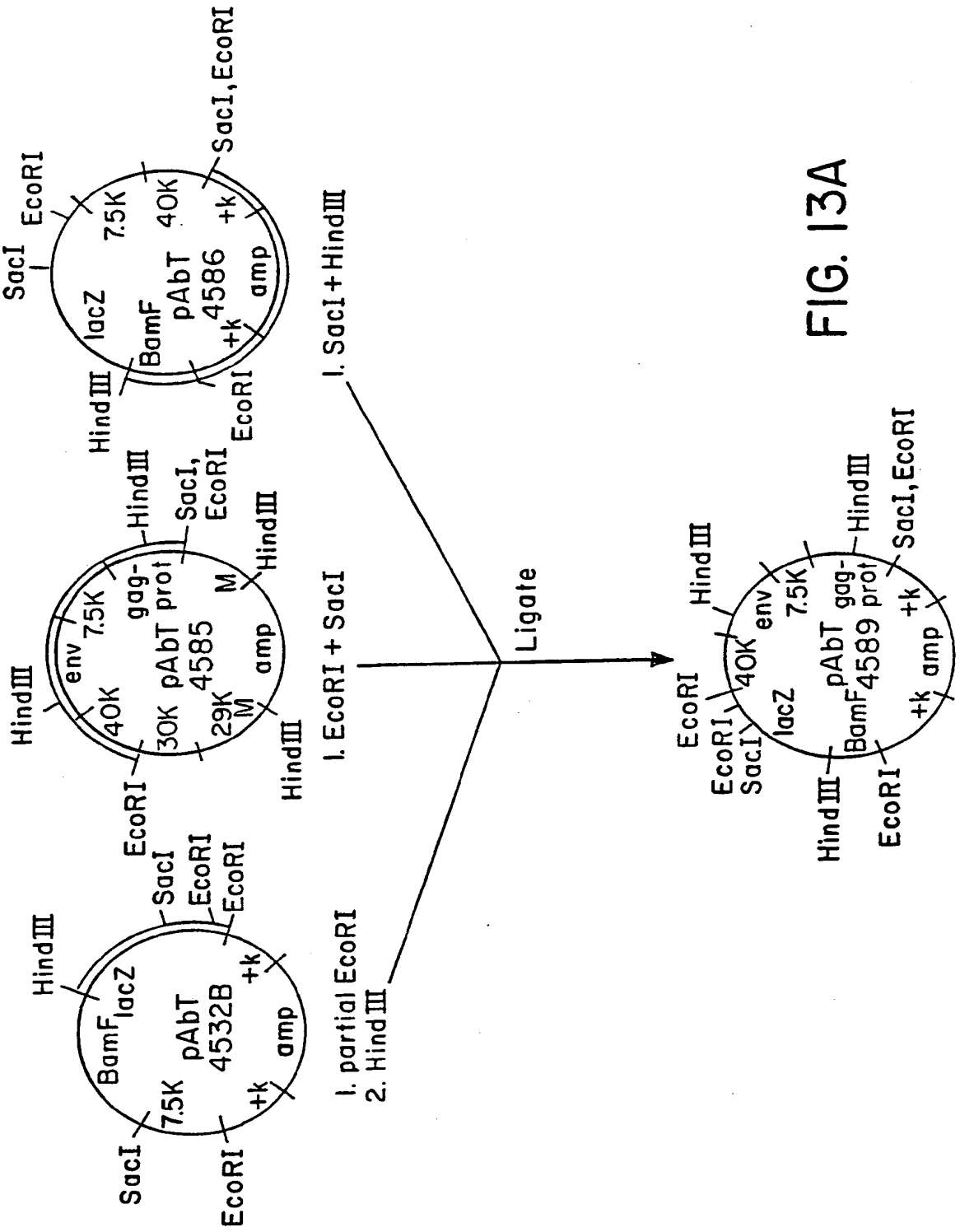


FIG. 13A

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02485

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC [4]: C 12 N 7/00, C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC [4]	C 12 N, C 12 P, G 01 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	GB, A, 2181435 (ONCOGEN) 23 April 1987 see pages 20-32 --	1-4,17,18
X	EP, A, 0243029 (UNITED STATES OF AMERICA) 28 October 1987 see the whole document --	1-4,17,18
X	EP, A, 0245136 (TRANSGÈNE S.A.) 11 November 1987 see the whole document --	1-4,17,18
X	Nature, volume 323, 25 September 1985, J.M. Zarling et al.: "T-cell responses to human AIDS virus in macaques immunized with recombinant vaccinia viruses", pages 344-346 see the whole article cited in the application	1-4,17,18
A	-- ./.	5-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th September 1989	19. 10. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

Form PCT/ISA/210 (second sheet) (January 1985)